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Multiple chemical exposures and its association with obesity and metabolic markers among women of reproductive age

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Abstract

Multiple chemical exposures and its association with obesity and metabolic markers among women of reproductive age

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Multiple classes of endocrine disrupting chemicals (EDCs) have been suspected as metabolism disrupting chemicals (MDCs) which can be defined as a particular class of EDCs that affect energy homeostasis. Although cumulative environmental epidemiological studies have reported the associations of EDCs with obesity and metabolic markers, information on the associations of multiple chemical exposures is limited. In addition, information on the associations with adiponectin and leptin, i.e., important adipokines, is also limited.

The current study aims to assess the associations of multiple chemical exposures with obesity and metabolic markers. Body mass index (BMI) and percent body fat were used as obesity markers. Two adipokines, i.e., adiponectin and leptin, γ-glutamyl transferase (GGT), fasting glucose, insulin, and homeostatic model assessment for insulin resistance (HOMA-IR) were used as metabolic markers. Women of reproductive age were recruited between 2015 and 2016. One spot urine, blood, and serum samples were collected within the same individuals. Associations of multiple classes of chemicals measured in different biological matrices with obesity and metabolic markers were assessed in each chapter. Non-persistent chemicals such as phthalate metabolites and environmental phenolics (Chapter 2) were

measured in urine samples. Heavy metals (Chapter 3) and persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated diphenyl ether (BDEs), and perfluorinated compounds (PFCs) (Chapter 4) were measured in whole blood and serum, respectively. Furthermore, overall associations of multi-pollutants (Chapter 4) were assessed and confirmed again in the last chapter.

Chapter 2 aims to assess the associations between urinary non-persistent chemicals including phthalate metabolites and phenolics (n = 459). Because most of chemicals showed significant correlations, I conducted elastic net penalized regression to select the most predictive variables to an outcome. Consistent significant associations of the chemicals in both single and multi-pollutant models after adjustment are as follows. Higher bisphenol A (BPA) levels were consistently associated with obesity markers and leptin. Higher ethyl paraben (EtP) and sum of di-(2-ethylhexyl) phthalate metabolites (ΣDEHPm) were associated with higher serum adiponectin. For fasting glucose, ΣDEHPm showed a positive association. Higher mono-isobutyl phthalate (MiBP) and bisphenol S (BPS) levels were associated with higher HOMA-IR. These findings indicate that MiBP, ΣDEHPm, BPA, and BPS may be the most predictive variables to obesity, fasting glucose, or insulin resistance in Chapter 2.

Chapter 3 aims to assess the associations between heavy metals, mercury (Hg), cadmium (Cd), and lead (Pb) (n = 456). Higher blood Hg was associated with higher BMI, GGT, and HOMA-IR, and lower adiponectin. Because Hg showed significant associations with several markers, mediation analysis was further conducted. Significant indirect effects of GGT and adiponectin were found in the association between blood Hg and HOMA-IR. Increased odds ratio of HOMA-IR>75th percentile per quartile increase of blood Pb was found, but the association disappeared after adjusting blood Hg which showed significant correlation with

blood Pb. The implication of this chapter is that possible mediators of IR induced by Hg exposure were suggested by conducting mediation analysis.

Chapter 4 aims to identify exposure patterns including urinary (Chapter 2), blood (Chapter 3), and persistent organic pollutants (POPs) such as polychlorinated biphenyl (PCBs), organochlorine pesticides (OCPs), and perfluorinated compounds (PFCs) using principal component analysis (PCA) and to confirm robustness of statistical analysis (n = 104). Because PCA can help to identify common exposures, distinct chemical groups such as PFCs and phthalates were found in PCA. Similar to Chapter 2, factor 4 characterized by phthalate metabolites showed positive significant associations with serum adiponectin and fasting glucose, which showed robustness of statistical analysis. Similar chemical classes such as PFCs and phthalates share metabolic pathway or common sources. Thus, management is needed to reduce these correlated chemical groups as a whole.

This study design is cross-sectional, and therefore, this study cannot provide causal inference. However, this study looked at the associations of multiple classes of chemicals with obesity and metabolic markers within the same individuals. The series of the studies showed that multiple chemicals were related to disruption in metabolism. The consistent associations of phthalate metabolites with fasting glucose and adiponectin assessed by different statistical methods and subgroups indicate that phthalate metabolites are the most predictive variables in this study population. Joint effects of multiple chemicals were not understood in this study. Therefore, the effects of multiple combinations of the chemicals are needed to confirm in experimental studies.

Keywords: multi-pollutants; obesity; adiponectin; leptin; fasting glucose; insulin resistance; phthalate; phenolics; heavy metals; persistent organic pollutant

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Contents

Abbreviation

BPA, bisphenol A;

BMI, body mass index;

BPS, bisphenol S;

BP1, benzophenone-1;

BP3, benzophenone-3;

Cd, Cadmium;

DEHP, di(2-ethylhexyl) phthalate;

DM, diabetes mellitus;

EtP, ethyl paraben;

GGT, γ-glutamiltransferase;

HOMA-IR, Homeostasis Model Assessment-Insulin resistance;

IR, insulin resistance;

MBP, mono-butyl phthalate;

MBzP, mono-benzyl phthalate;

MCMHP, mono(2-carboxymethylhexyl) phthalate;

MECPP, mono(2-ethyl-5-carboxypentyl) phthalate;

MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate;

MEOHP, mono(2-ethyl-5-oxohexyl) phthalate;

MEP, mono-ethyl phthalate;

MDCs, metabolism disrupting chemicals;

MeP, methyl paraben;

MiBP, mono-isobutyl phthalate;

MMP, mono-methyl phthalate;

OLS, ordinary least squares;

OCPs, organochlorine pesticides;

Pb, lead;

PBDEs, polybrominated diphenyl ether;

PCBs, polychlorinated biphenyl;

PFCs, perfluorinated compounds;

PPARγ, peroxisome proliferator-activated receptor γ;

PrP, propyl paraben;

TCS, triclosan;

T2DM, type 2 diabetes mellitus;

%BF, percent body fat;

Chapter 1 Background

1.1 Obesity and metabolic markers

Obesity and metabolic markers

Obesity is accompanied by increased oxidative stress and several comorbidities such as type II diabetes mellitus, and dyslipidemia (Fernández-Sánchez et al., 2011; Fingeret et al., 2018). Thus, obesity and related health effect markers, such as adiponectin and leptin, γ-glutamyl transferase (GGT), and insulin resistance (IR), measured in this study are closely linked with each other (Wu et al., 2011; Yadav et al., 2013).

The term ‘adipokine’ is used as any substance released by adipose tissue, and adipose tissue is now regarded as complex metabolic endocrine organ which releases adipokines such as adiponectin and leptin (Fantuzzi, 2005; Yadav et al., 2013). Leptin is a well-known adipokine which can modulate energy expenditure by involving satiety in hypothalamus and caloric intake reduction, and has been reported to be positively associated with body mass index (BMI), a surrogate marker of obesity (Yadav et al., 2013). Contrary to other adipokines, adiponectin has an inverse relationship with obesity (Yadav et al., 2013). Adiponectin has a regulatory role in reducing IR (Ricci and Bevilacqua, 2012; Yadav et al., 2013).

Serum γ-GGT has been usually used as a marker of excessive alcohol consumption or liver dysfunction in clinical field (Teschke et al., 1977), and it has been also suggested as a marker of oxidative stress (Lee et al., 2004). In addition to malondiadehyde (MDA) and 8-hydroxy-deoxyguanosine (8-OHdG), γ-GGT has been interpreted as a non-specific oxidative stress marker in environmental epidemiological studies (Dong et al., 2018).

The relationship between the markers measured in this study has been reported (Bastard et al., 2006; Stefan, 2002; Thamer et al., 2005; Yamamoto et al., 2004). Based on previous literature, some markers can be on the causal pathway to other markers. Low adiponectin levels were reported to be related to type 2 diabetes mellitus (T2DM) and IR (Bastard et al., 2006). It was previously reported that a decline in adiponectin precedes IR (Stefan, 2002; Yamamoto et al., 2004). For GGT, non-specific marker of oxidative stress was reported to predict reduced insulin sensitivity, which might be related to hepatic IR (Thamer et al., 2005). Lastly, obesity has been closely linked with insulin resistance, and the mechanisms including via adipokines such as adiponectin have been suggested (Hardy et al., 2012). However, which one precedes the other one is unclear. It was previously reported that obesity is accompanied by increased oxidative stress (Fernández-Sánchez et al., 2011) and IR (Hardy et al., 2012) which commonly precedes T2DM. Although it has been commonly recognized that obesity can induce IR, whether obesity precedes IR or vice versa is a matter of debate (Erion and Corkey, 2017). Thus, relationship between these markers is needed to interpret findings with caution.

1.2 Multiple chemical exposures

In the last several decades, most of epidemiological studies on the health effects of chemical exposure have focused on evaluating the effect of individual chemicals. However, real human populations are exposed to multiple chemical mixtures, and therefore, multiple chemical exposures are of increasing concern. Number of detects of chemicals in the present study population is suggested in Fig. 1-1. Due to high cost, multiple chemical exposure studies have limited feasibility on collecting and measuring exposure markers, in particular in different kind of media such as urine and blood, in the same individual. In addition to high cost, statistical analysis is another important challenge in environmental epidemiology. Due to complexity among chemicals, it is difficult to interpret the findings.

Multi-pollutant exposure has long been recognized as an important issue in environmental epidemiology and toxicology. However, considering the importance, risk assessment and bioanalytical tools of evaluating mixtures have been underdeveloped. Mixture toxicity of chemicals has been tested in experimental studies, but most of these tests are restricted to a low number of combinations or single receptor in *in vitro* studies (Altenburger et al., 2018). However, a number of adverse outcome pathways (AOP) describing links as a series of causally connected multiple molecular events (http://www.aopwiki.org). In addition, human risk assessment has been generally derived from toxicity data of individual chemical from animal models (Persad and Cooper, 2008). Epidemiological studies on multi-pollutant exposure with refined approaches may complement the toxicology data and be utilized in the risk assessment.

As concerns on multi-pollutant exposure have increased, in recent years, environmental epidemiological studies have considered multiple chemical exposures (Patel et al., 2010; Rosofsky et al., 2017; Mustieles et al., 2017). In 2010, associations between 266 exposures

and T2DM were evaluated using NHANES data (Patel et al., 2010). Patel et al. introduced an Environmental-Wide Association Study (EWAS) which is interpreted in a manner analogous to a Genome Wide Association Study (GWAS). Though the EWAS study corrected multiple comparisons, co-exposure were not adjusted. Assessment of effects of multi-pollutant is a challenge not only in environmental epidemiology, but also in environmental toxicology. Several methods such as dimension reduction, shrinkage, variable selection, and statistical learning have been suggested and performed in multiple chemical exposure studies (Lazarevic et al., 2019). One efficient approach which considers multi-pollutant exposure is adjustment for covariate by co-exposure. Considering the importance of multi-pollutant exposure, comparative association studies considering the multi-pollutants in multiple biological matrices are warranted.

Fig. 1-1. Number of chemical exposure markers detected in each study participant (n = 104). Each bar represents one individual. The figure includes only study participants with urine, whole blood, and serum samples.

1.3 Effects of chemical exposure on obesity and metabolic markers

In 2006, Grün and Blumberg coined ‘obesogen’ which means a xenobiotic chemical that can disrupt the normal developmental and homeostatic controls over adipogenesis and energy balance (Grün and Blumberg, 2006). Recently, a more extended term, ‘metabolism disrupting chemicals (MDCs)’ has been used (Heindel et al., 2017; Nadal et al., 2017; Mimoto et al., 2017), which reflects an increasing interest and concern on the effects of chemicals. MDCs have been reported to induce obesity, T2DM, and non-alcoholic fatty liver disease (NAFLD) (Heindel et al., 2017). Chemicals which can act as MDCs measured in this study were briefly summarized in Table 1-1. Cumulative studies on relationship of chemical exposure and metabolic effects have been reported. Multiple classes of chemicals with short to long half-lives have been suspected as MDCs. Multiple MDCs can affect different receptors and target sites (Heindel et al., 2017; Nadal et al., 2017; Mimoto et al., 2017). For example, several phthalates such as di-(2-ethylhexyl) phthalate (DEHP) are known as peroxisome proliferator-activated receptor γ (PPARγ) agonists (Zhang et al., 2017), and experimental and human studies reported relationship of phthalate exposure with insulin resistance or T2DM (Lind et al., 2012; James-Todd et al., 2016; Song et al., 2016; Zhang et al., 2017). Heavy metals such as mercury (Hg), cadmium (Cd), and lead (Pb) have been reported to induce oxidative stress which plays an important role in insulin resistance (Tinkov et al., 2015; González-Villava et al., 2016). Perfluorinated compounds (PFCs), one of the persistent organic pollutants (POPs), such as perfluorooctane sulfonic acid (PFOS) are known as a PPARα agonist (Abbott, 2009), and PFOS was reported to induce glucose tolerance and increased insulin levels in an animal study (Lv et al., 2013). Cumulative previous studies including experimental and epidemiological studies have reported metabolic

effects of chemicals. However, only limited information on relationship of chemical exposure with adipokines exists. Moreover, most of previous studies focused on metabolic effects of single pollutant chemical exposure, and MDCs have been related to disruption in multiple endpoints within same individuals (Heindel et al., 2017). Thus, it is needed to evaluate effects of multiple chemical exposure with several metabolic markers

Table 1-1. Metabolic effects of chemicals and their half-lives.

Chemical group	Compound	Experimental studies	Epidemiological studies	References
Phthalates	DEHP, MEHP, MEP, etc.	-*In vitro*: · PPARγ agonist · reduced glucose-stimulated insulin secretion (GSIS), insulin content and increased ROS in β-cells -*In vivo*: insulin resistance, reduced hepatic glycogen, and increased reactive oxygen stress (ROS)	- T2DM · Increased odds ratio (OR) in the highest quartile compared to the first quartile: vMiBP and MMP; Sweden; aged 70 yr; cross-sectional study vMBzP; NHANES 2001-2008; women aged 20-79 yr; cross-sectional study vsum of MBP and MiBP, sum of DEHP metabolites, MBP, and MiBP; Nurses' Health Study (NHS); mean age, 65.6 yr; prospective investigation -IR · Positive association vMBP, MEHP, and MEP; NHANES 1999-2002; adults; cross sectional study vMiBP and MMP; Sweden; aged 70 yr; cross-sectional study vMiBP; NHANES 2001-2008; women aged 20-79 yr; cross-sectional study	Campioli et al., 2014; Lind et al., 2012; Martinelli et al., 2006; James-Todd et al., 2016; Rajesh et al., 2013; Rajesh et al., 2014; Stahlhut et al., 2007; Sun et al., 2015; Zhang et al., 2017
Environmental phenols (Bisphenols, benzophenone, antimicrobials, and parabens)	BPA	-*In vitro*: · weak estrogen receptor (ER) agonist · reduced GSIS and increased ROS in β-cells - *In vivo*: glucose intolerance	- T2DM · Increased odds ratio (OR) in the highest quartile compared to the first quartile: vBPA; China; median age, 59.0 yr; cross-sectional study vBPA; 1.68 (1.23, 2.30); NHANES 2003-2008; mean age, 44.3 (men) and 45.6 (women) yr; cross-sectional study vBPA; 2.08 (1.13, 1.87); NHSII; mean age, 45.6 yr; prospective investigation -IR · Positive association vBPA; NHANES; age 18-74 yr; corss-sectional study vBPA; BMI<24 kg/m^2; China; mean age, 60.8 yr; cross-sectional study	Aloso-Magdalena et al., 2010; Batista et al., 2012; Bodin et al., 2014; Kim and Park, 2013; Moon et al., 2015; Indumathi et al., 2013; Lang et al., 2008; Ohlstein et al., 2014; García-Arévalo et al., 2016; Ning et al., 2011; Sakurai et al., 2004; Shankar and Teppala, 2011; Song et al., 2012; Sun et al., 2014; Valentino et al., 2013; Wang et al., 2012; Xin et al., 2014;
Heavy metals	Hg	- *In vitro*: · reduced GSIS, increased ROS, PI3 kinase and Akt, induced apoptosis and necrosis in β-cells	- T2DM: · Increased hazard ratio (HR) in the highest quantile compared to the lowest quantile): vHg; American young adults aged 20-32 yr; prospective study	Chen et al., 2006; Chen et al., 2010; He et al., 2013; Tinkov et al., 2015
	Pb	- *In vitro*: oxidative stress		Chen et al., 2009; Gonzalea-Villava et al., 2016

	Cd	- *In vitro*: reduced GSIS, increased ROS, mitochondrial dysfunction, apoptosis, mediated by JNK in β-cells - *In vivo*: insulin resistance, increased insulin levels		Chang et al., 2013; Han et al., 2003; El Muayed et al., 2012; Treviño et al., 2015
Persistent organic pollutants (POPs)	PCBs	- *In vitro*: increased insulin secretion and Ca^{2+} signaling in β-cells - *In vivo*: glucose intolerance	-T2DM · Increased incidence density ratio in the highest quartile compared to the first quartile: vPCBs: significant, positive association in women, but not in men; US; adults; cohort study vPCBs: no association; USA; mean age, 52.2 yr (DM), 47.9 (no DM); cohort study	Baker et al., 2013; Fischer et al., 1999; Gray et al., 2013; Turyk et al., 2009; Vasiliu et al., 2006
	PFOA, PFOS	- *In vitro*: PPARα agonist - *In vivo*: altered lipid metabolism		Abbott, 2009; Beggs et al., 2016; Hines et al., 2009; Wan et al., 2014; Yan et al., 2015

This table was revised from Mimoto et al., 2017.

1.4 Study design and objectives

The aim of this study is to investigate the associations of multiple chemical exposure with obesity and metabolic markers, i.e., GGT, adiponectin, leptin, fasting glucose, and HOMA-IR, among women of reproductive age. To this end, multiple classes of chemicals and health effect markers were measured in the same study population. Because this study is cross-sectional study, one spot urine, whole blood, and werum samples were collected.

This study consists of three parts (Fig. 1-2). In the first chapter (Chapter 2), the associations between non-persistent chemicals measured in urine and obesity and its related health effect markers were assessed. In Chapter 3, the associations of heavy metals measured in whole blood were assessed. In the last chapter (Chapter 4), the associations of multi-pollutant inclduing urinary (Chpater 2), blood (Chpater 3), and serum chemicals were assessesd. The analysed chemicals were listed in Table 1-2. Furthermore, multiple chemicals including chemicals used in the last two chapters were again included to comfirom robustness of the analysis.

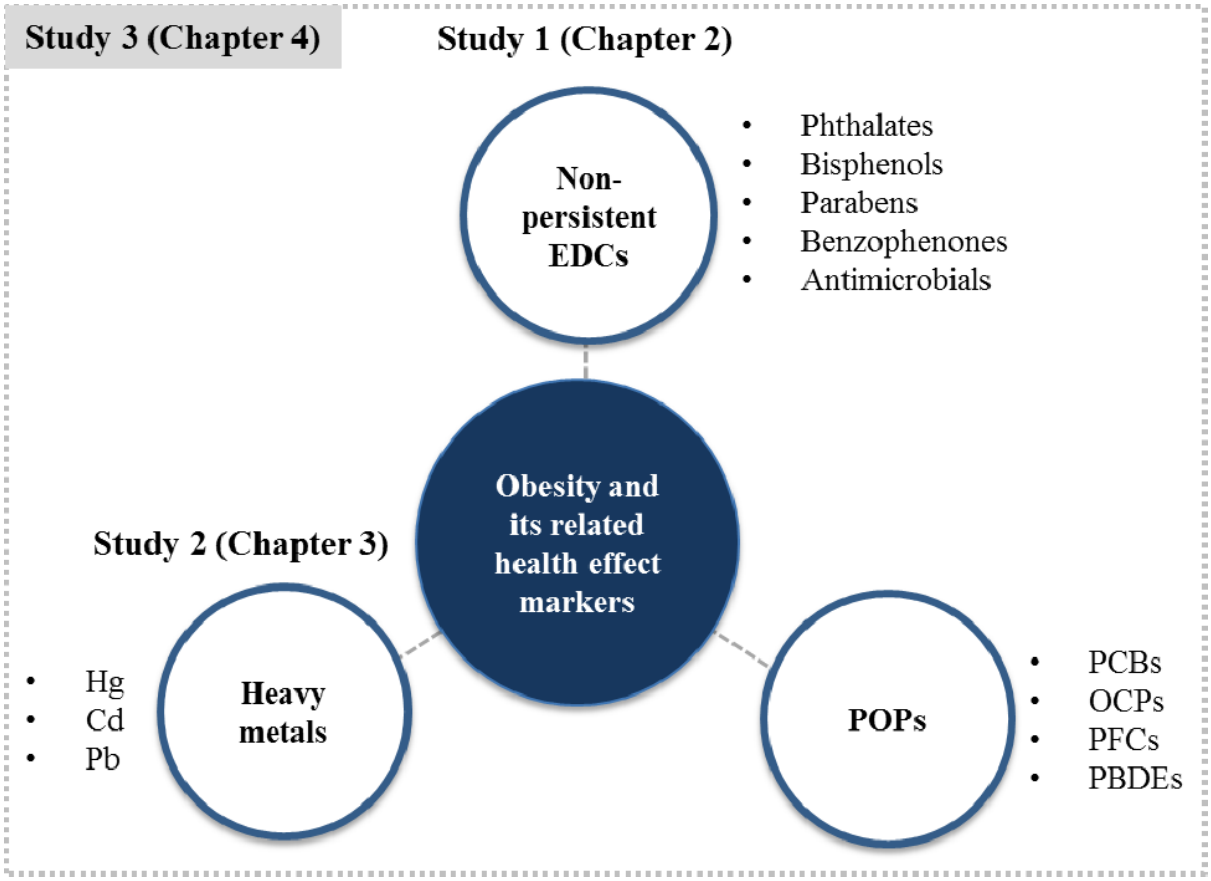

Fig. 1-2. Study design to investigate the associations between multipe chemical exposure and obesity and its related health effect markers.

Table 1-2. Multiple chemicals measured in this study.

Biological matrix	Chemical class	Chemical
Urine	Phthalate metabolites	**Monomethyl phthalate (MMP)**
		Mono(3-carboxypropyl) phthalate (MCPP)
		Monoethyl phthalate (MEP)
		Mono-isopropyl phthalate (MiPrP)
		Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)
		Mono-[(2-carboxymethyl)hexyl] phthalate (MCMHP)
		Mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP)
		Mono(2-ethyl-5-oxohexyl)phthalate (MEOHP)
		Mono-2-isobutyl phthalate (MiBP)
		Mono-n-butyl phthalate (MBP)
		Monobenzyl phthalate (MBzP)
		Monocyclohexyl phthalate (MCHP)
		Mono-n-pentyl phthalate (MPeP)
		Monohexyl phthalate (MHxP)
		Mono(2-ethylhexyl)phthalate (MEHP)
		Monooctyl phthalate (MOP)
		Mono-isononyl phthalate (MiNP)
		Mono-isodecyl phthalate (MiDP)
	Bisphenol analogues	**Bisphenol S (BPS)**
		Bisphenol F (BPF)
		Bisphenol A (BPA)
		Bisphenol B (BPB)
		Bisphenol AF (BPAF)
		Bisphenol AP (BPAP)
		Bisphenol Z (BPZ)
		Bisphenol P (BPP)
	Parabens	**Methyl paraben (MeP)**
		Ethyl paraben (EtP)
		Propyl paraben (PrP)
		Butyl paraben (BuP)
		Heptyl paraben (HeP)
		Benzyl paraben (BzP)
		Methyl protocatechuate (OH-MeP)
		Ethyl protocatachuate (OH-EtP)
		3,4-dihydroxy benzoic acid (3,4-DHB)[a]
		4-hydroxybenzoic acid (4-HB)[a]
	Benzophenones	**Benzophenone-1 (BP-1)**
		Benzophenon-3 (BP-3)
		Benzophenon-8 (BP-8)
		4-hydroxybenzophenone (4OH-BP)[a]
	Antimicrobials	**Triclosan (TCS)**
		Triclocarban (TCC)
Whole blood	Heavy metals	**Mercury (Hg)**
		Cadmium (Cd)
		Lead (Pb)

Serum	PCB	**CB 52**
		CB 118
		CB 138
		CB 153
		CB 180
		CB 187
	OCP	**HCB**
		b-HCH
		oxy-CHL
		trans-NonaCHL
		***p,p'*-DDE**
		***p,p'*-DDT**
	BDE	BDE-28
		BDE-47
		BDE-99
		BDE-100
		BDE-153
		BDE-154
		BDE-183
	PFCs	Perfluorobutane sulfonic acid (PFBS)
		Perfluorohexane sulfonic acid (PFHS)
		Perfluorooctane sulfonic acid (PFOS)
		Perfluorodecane sulfonic acid (PFDS)
		Perfluoropentanoic acid (PFPeA)
		Perfluorohexanoic acid (PFHxA)
		Perfluoroheptanoic acid (PFHpA)
		Perfluorooctanoic acid (PFOA)
		Perfluorononanoic acid (PFNA)
		Perfluorodecanoic acid (PFDA)
		Perfluoroundecanoic acid (PFUnDA)
		Perfluorododecanoic acid (PFDoDA)
		Perfluorotridecanoic acid (PFTrDA)
		Perfluorotetradecanoic acid (PFTeDA)
		Perfluorohexadecanoic acid (PFHxDA)
		Perfluorooctadecanoic acid (PFOcDA)

Bold characteristics indicate chemicals with detection frequency (DF) greater than 70%.

Chemicals with ≥70% DF were included in statistical analysis.

[a]Non-specific metabolites were excluded in statistical analysis.

Chapter 2 Associations of urinary non-persistent chemicals with obesity and metabolic markers

2.1 Introduction

Diabetes mellitus (DM) has become a major global health challenge. Between 1980 and 2014, the global age-standardized diabetes prevalence increased from 5.0% to 7.9% in females, and from 4.3% to 9.0% in males (NCD Risk Factor Collaboration, 2016). T2DM has a multifactorial etiology (Zheng et al., 2018). Genetic predisposition and lifestyle habits such as diet, physical activity, and smoking are all important contributors to this disease etiology (Zheng et al., 2018). Chemical exposure also has been suggested to be an important contributing factor, although the detailed mechanisms underlying the development of T2DM-related metabolic disturbances are not clearly understood (Heindel et al., 2017; Mimoto et al., 2017; Song et al., 2016).

An increasing number of experimental observations suggest the involvement of chemicals in the etiology of this disease. In rats, exposure to di-(2-ethylhexyl) phthalate (DEHP) induced liver damage, glucose tolerance, and insulin resistance (Zhang et al., 2017). One potential mechanism explaining this relationship is that these chemicals produced peroxisome proliferator-activated receptor γ (PPARγ)-related metabolic disturbances (Zhang et al., 2017), and several phthalates and environmental phenolics can act as PPARγ agonists (Hurst and Waxman, 2003; Pereira-Fernandes et al., 2013). Chemicals like DEHP may affect the pancreas and alter glucose homeostasis in rats (Lin et al., 2011). Adipokines such as adiponectin and leptin play important roles in the etiology of insulin resistance or T2DM (Yadav et al., 2013). While serum adiponectin is known to be a marker of PPARγ activation

in vivo (Yang et al., 2004), to date, studies that have investigated adipokine levels in relation to adverse metabolic effects of DEHP among healthy adults are rare, leaving a significant gap in our understanding of metabolic effects.

Several studies from around the world have suggested that in humans, chemicals such as phthalates and environmental phenolics contribute to T2DM-related metabolic disturbances, e.g., dysregulated glucose homeostasis and insulin resistance (Heindel et al., 2017; Mimoto et al., 2017; Song et al., 2016). In a recent meta-analysis, higher urinary concentrations of bisphenol A (BPA) and phthalates were found to be associated with the increased prevalence of T2DM and other related metabolic traits (Song et al., 2016). In addition, it is noteworthy that most of the association studies that have been conducted in humans have only considered a handful of chemicals in terms of their health effects. Since humans are exposed to a myriad of pollutants, and many chemicals may be related to the development of T2DM, an epidemiological study design that allows for multiple chemicals as determinants for a given disease is necessary. Recently, multi-pollutant exposure approaches have been introduced by a number of epidemiological studies, and have identified several chemical determinants of the given health effects they were examining. For example, multiple environmental factors for T2DM were identified from dozens of chemicals that were measured among the general population of the US (Patel et al., 2010). In addition, a study by Mustieles et al. used a multi-pollutant approach using an elastic net penalty to select predictors among persistent organic pollutants (POPs) in adipose tissue and identified chemicals that were consistently associated with metabolic disorders (Mustieles et al., 2017).

In the present study, aim of this study is to identify chemicals that are associated with metabolic markers including adipokines, glucose, and insulin measured in serum in premenopausal adult women. For this purpose, dozens of chemicals measured in the urine of healthy adult females in Korea were assessed for associations with metabolic markers using

both single- and multi-pollutant models. The results of this study will help identify chemicals that may potentially play roles in IR in the general adult female population. This study will also generate hypotheses that can be tested in other human populations or experimental models in the future.

2.2 Materials and methods

2.2.1 Study population and sample collection

Women of reproductive age (20-48 years old) (n = 516) were recruited from five university hospitals located in Seoul, Incheon, Ansan, and Jeju, South Korea between 2015 and 2016. Spot blood and urine samples were collected from the participating women. Participants were fasted for at least nine hours before the visit to hospitals for the blood and urine sampling. EDTA-treated fasting blood and urine samples were stored in polypropylene cryovials and tubes at -80°C and -40°C, respectively, until analysis. Participants completed a questionnaire at the time of recruitment providing information on their sociodemographic characteristics, alcohol consumption, and sources of exposure to environmental chemicals.

Two participants who had fasting glucose measurements ≥126 mg/dL and 33 participants who were pregnant at the time of recruitment were excluded. Participants missing information about their age, BMI, urinary nicotine metabolite level, and/or current alcohol consumption habits and participants without urine or blood samples were also excluded. The final number of subjects included in the study was 459. Informed consent was obtained from all participants. This study was approved by the Institutional Review Board of the School of Public Health, Seoul National University (IRB No. 1509/001-011).

2.2.2 Measurement in urine and blood samples

Health effect markers

Urinary creatinine, serum glucose, and serum insulin were measured by a commercial laboratory (Green Cross LabCell, Yongin, Korea). Serum adiponectin and leptin were measured using an enzyme-linked immunosorbent assay kit (Duoset, R&D Systems, Minneapolis, MN, USA). Insulin resistance status was estimated using the homeostatic model

assessment for insulin resistance (HOMA-IR) and was calculated as fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5 (Wallace et al., 2004).

Chemical analyses

Sample preparation and instrumental analyses

Target compounds including phthalate metabolites and environmental phenols were analyzed from urine samples following methods modified from previous studies (Asimakopoulos et al., 2014a; Gao et al., 2016). Urine samples were measured for phthalate metabolites including mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-isopropyl phthalate (MiPrP), mono-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), mono-n-pentyl phthalate (MPeP), monocyclohexyl phthalate (MCHP), monohexyl phthalate (MHxP), mono-benzyl phthalate (MBzP), mono(3-carboxypropyl) phthalate (MCPP), monooctyl phthalate (MOP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-carboxymethylhexyl) phthalate (MCMHP), mono-isononyl phthalate (MiNP), mono-isodecyl phthalate (MiDP) and environmental phenols including bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), bisphenol B (BPB), methyl paraben (MeP), ethyl paraben (EtP), propyl paraben (PrP), butyl paraben (BuP), benzyl paraben (BzP), benzophenone-1 (BP1), benzophenone-3 (BP3), triclosan (TCS), and triclocarban (TCC). Phthalate metabolites were purchased from AccuStandard (New Haven, CT, USA) and Cambridge Isotope Laboratories (Andover, MA, USA). The isotope-labeled internal standards for the phthalate metabolites were purchased from Cambridge Isotope Laboratory. Environmental phenols and their internal standards were also purchased from Cambridge Isotope Laboratories.

Pretreatment procedures of the urinary phthalate metabolites were conducted as follows. 200 μL of ammonium acetate buffer (1.0 M, pH = 4.5), including β-glucuronidase (2 μL/mL), were added to 500 μL of the urine samples spiked with ^{13}C-labeled internal standards. The urine samples were incubated at 37 ℃ for 12 hours. Phthalate metabolites were extracted using a solid phase extraction (SPE) setup. The SPE cartridges (ABS ELUT-Nexus, Varian, Walnut Creek, CA, USA; 60 mg/3 mL) were conditioned with 1.5 mL of acetonitrile and 1.2 mL of phosphate buffer (pH = 2). The urine samples were diluted with 1 mL of phosphate buffer and loaded into the SPE cartridges. The SPE cartridges were washed with 2.0 mL of formic acid (0.1 M) and 1.2 mL of Milli-Q water and dried with nitrogen. Target compounds were eluted from the dried cartridges with 1.2 mL of acetonitrile and 1.1 mL of ethyl acetate. The extract was nearly dried under a nitrogen stream and was resolved in 0.5 mL of a mixture of acetonitrile and Milli-Q water in a 1:9 ratio for instrumental analysis.

The environmental phenols, including bisphenol analogues, parabens, benzophenones and triclosan, in the urine samples were prepared as follows. Internal standards were added to 500 μL of each urine sample. 250 μL of ammonium acetate (1.0 M, pH = 4.5) with β-glucuronidase (2 μL/mL) was added to the urine samples, then incubated at 37 °C for 12 hours. The urine samples were mixed with 3.0 mL ethyl acetate and were mechanically shaken for 60 min, then centrifuged at 4000 rpm for 10 min. The supernatant was transferred to a new tube. After repeating this step two times, the combined supernatant was washed with 1 mL of Milli-Q water. The extract was nearly dried under a nitrogen stream and resolved in 0.5 mL of methanol for the instrumental analyses.

Phthalate metabolites and environmental phenols were chromatographically separated using an Agilent 1260 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Betasil C18 column (Thermo Electron, Bellefonte, PA; 100 mm × 2.1 mm, 5

μm). Target compounds in the urine samples were quantified using an API 4000 electrospray triple quadrupole mass spectrometer (ESI–MS/MS; AB Sciex, Framingham, MA, USA).

Quality assurance and quality control

In the sample treatment step, two procedural blank and matrix-spiked samples were processed along with the real samples. The midpoint calibration standard was injected every 10 to 15 samples to monitor instrumental sensitivity. The procedural blank concentrations of the target compounds were subtracted from those of the urine samples. The limits of quantification (LOQs) of the phthalate metabolites and environmental phenols ranged from 0.01 to 0.05 ng/mL. Recoveries of phthalate metabolites, bisphenols, benzophenones, parabens, and antimicrobials were 83-122%, 97-111%, 69-127%, 107-131%, and 120-147%, respectively, in the blanks. The recoveries of phthalate metabolites, bisphenols, benzophenones, parabens, and antimicrobials were 62-205%, 89-104%, 63-119%, 83-99%, and 112-131% respectively, in the matrix blanks.

2.2.3 Statistical analysis

Only chemicals that were detected in >70% of the population were considered for the statistical comparisons. A proxy value (the LOQ divided by square root of 2) was used to replace the nondetectable values (Hornung et al., 1990). Spearman correlation analyses were conducted to identify correlations between creatinine-corrected chemical concentrations in the urine and the metabolic markers.

Associations between urinary chemical levels and metabolic markers were investigated in two steps. First, for each urinary chemical, ordinary least squares (OLS) regression was conducted with appropriate covariates included in the models. Second, to prevent problems associated with multicollinearity and overfitting in the model, elastic net penalized

regressions with 10-fold cross validations were performed to select relevant predictors of certain metabolic markers, among the chemicals measured. Then, with the chemicals selected using the elastic net regression, ordinary least squares (OLS) regression models were used to obtain unpenalized, mutually adjusted coefficient estimates.

Before data analyses, creatinine-corrected concentrations of the chemicals were ln-transformed because they had a right-skewed distribution. For all regression models, the ln-transformed concentrations were mean-centered, divided by two standard deviations, and adjusted for the same covariates, i.e., age (continuous), urinary nicotine metabolite (categorical: <LOQ, >LOQ-500, ≥500 ng/ml), and current alcohol consumption habits (categorical: drinker or nondrinker). Information on age and current alcohol consumption was obtained from questionnaire. Threshold value of smoking (urinary nicotine metabolite ≥500 ng/ml) was based on a previous study (Apseloff et al., 1994). These covariates were chosen based on previous studies (Zheng et al., 2018). These covariates were also selected in one or more elastic net regression models in the present study.

A value of $p<0.05$ was considered to be significant. All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA) except the Spearman correlation analyses. The correlation matrix was calculated using R version 3.5.1 and visualized using the R package ‘corrplot’.

2.3 Results

2.3.1. Study population and urinary levels of measured chemicals

Study participants were healthy adult females between the ages of 20 and 48 years old. The majority (65.7%) showed BMI values within the normal range (Table 2-1). The majority (66.5%) was current consumers of alcohol, but about 10% was reported to be passive or active smokers. Levels of the measured metabolic markers are summarized by age group, BMI category, pregnancy status, level of urinary nicotine metabolite, and current alcohol consumption (Table 2-1).

Urinary concentrations of phthalate metabolites and phenolics that were detected in greater than 70% of the study population are shown in Table 2-2. The urinary correlations of MeP and PrP (creatinine-corrected) showed relatively high correlation (ρ=0.67); those between MiBP, MBP, ΣDEHPm, and MBzP concentrations were moderately correlated (Fig. 2-1).

Table 2-1. Characteristics of study population (n = 459).

	N (%)
Age (years)	
20-29	125 (27.2)
30-39	224 (48.8)
40-48	110 (24.0)
Body mass index (kg/m^2)	
<18.5	28 (6.1)
18.5-22.9	293 (63.8)
23.0-24.9	67 (14.6)
25.0-29.9	56 (12.2)
≥30.0	15 (3.3)
Urinary nicotine metabolite (ng/mL)	
<10	410 (89.3)
11-499	24 (5.2)
≥500	25 (5.5)
Current alcohol drinking	
Drinker	322 (70.2)
Non-drinker	137 (29.9)
	Median (25th-75th)
Percent body fat (%) (n = 298)	25.6 (29.3-33.5)
Adiponectin (μg/mL)	6.6 (5.4-7.8)
Leptin (ng/mL)	9.6 (5.8-15.5)
GGT (U/L)	12 (10-17)
Fasting glucose (mg/dL)	88 (83-93)
Fasting insulin (μU/mL)	6.1 (4.0-8.6)
HOMA-IR	1.3 (0.9-1.9)

Table 2-2. Concentrations of phthalate metabolites and phenolics in urine samples collected from participating women (n = 459, unit: ng/mL).

		Detection frequency	Percentiles		
			P25	P50	P75
Phthalate compounds	**Metabolites**				
DMP	**MMP**	98.7	0.82	1.69	3.51
DEP	**MEP**	100.0	2.62	5.78	15.53
DiPrP	**MiPrP**	14.6	-	-	-
DBP	**MBP**	98.9	2.61	4.91	7.66
DiBP	**MiBP**	94.6	0.87	2.07	3.69
DPeP	**MPeP**	6.8	-	-	-
DCHP	**MCHP**	2.6	-	-	-
DHxP	**MHxP**	33.1	-	-	0.08
BBzP	**MBzP**	97.6	0.23	0.50	0.96
DOP	**MCPP**	46.6	-	-	0.66
	MOP	25.9	-	-	0.08
DEHP	**MECPP**	100.0	5.47	11.33	19.59
	MEHHP	99.6	1.42	2.53	4.06
	MEOHP	99.3	0.72	1.27	2.06
	MCMHP	99.8	2.19	3.91	6.84
	ΣDEHPm[a]	-	10.76	20.35	32.09
DiNP	**MiNP**	44.4	-	-	0.08
DiDP	**MiDP**	8.9	-	-	-
Environmental phenols					
Bipsphenols	**BPA**	97.6	0.27	0.51	0.92
	BPS	83.7	0.03	0.08	0.24
	BPF	3.7	-	-	-
	BPB	1.3	-	-	-
	BPAP	4.8	-	-	-
Parabens	**MeP**	100.0	13.20	46.10	124.70
	EtP	98.0	4.00	17.50	48.90
	PrP	93.7	0.30	2.30	10.10
	BuP	64.5	-	0.10	0.60
	BzP	1.3	-	-	-
Benzophenones	**BP1**	98.3	0.64	1.21	2.63
	BP3	73.2	0.02	0.43	1.65
	BP8	23.3	-	-	-
Antimicrobials	**TCS**	89.5	0.10	0.30	0.90
	TCC	3.9	-	-	-

[a]ΣDEHPm indicates the sum of the DEHP metabolites including MECPP, MEHHP, MEOHP, and MCMHP.

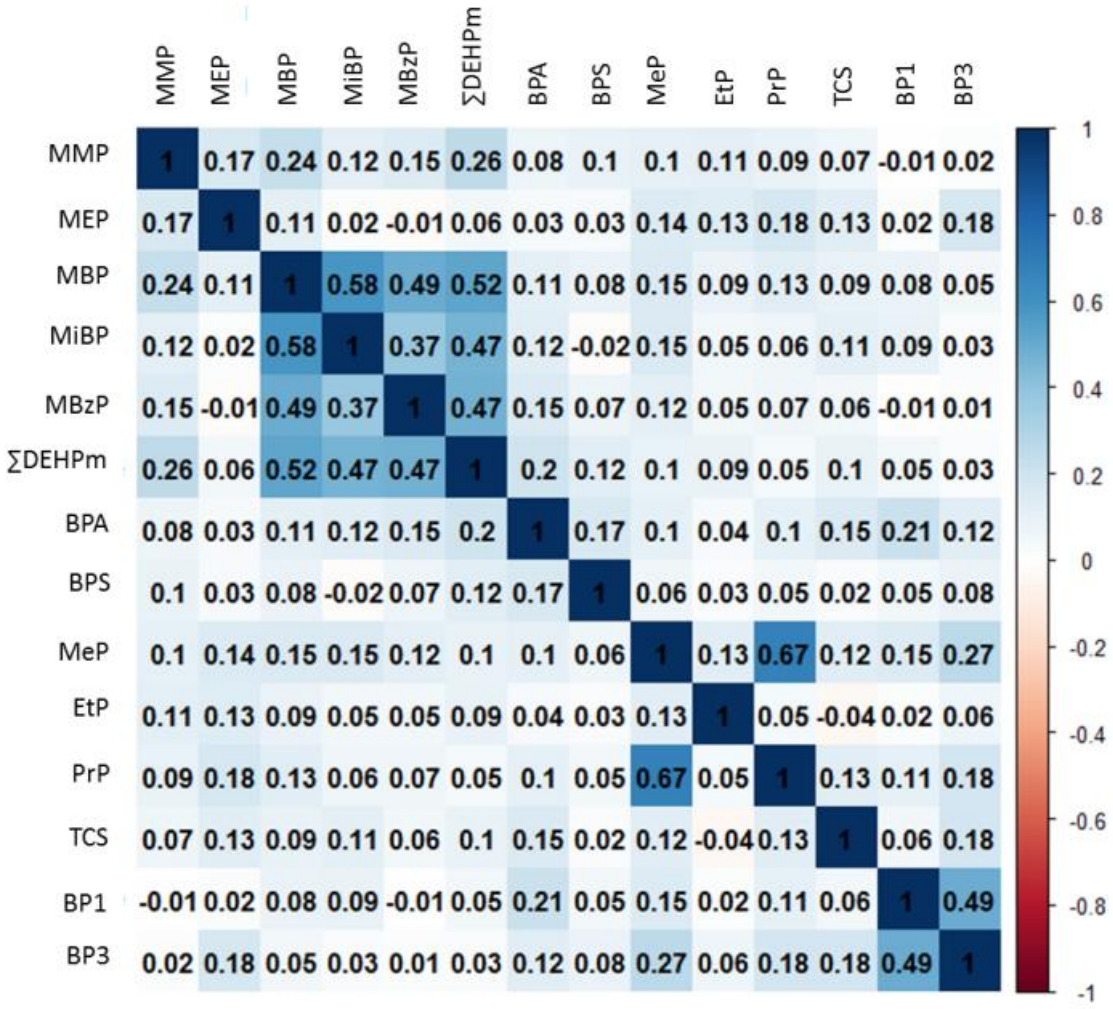

Fig. 2-1. Spearman's correlation coefficients between creatinine-corrected concentrations of chemicals in urine samples (n = 459).

2.3.2. Associations with obesity and metabolic markers

In the single and multi-pollutant models, several urinary chemicals were identified as significant determinants of obesity and metabolic markers, and are shown in Tables 2-3 and 2-4, respectively. The β and 95% CI of the predictive chemicals in multi-pollutant models are as follows. The urinary concentrations of BPA showed consistent positive associations with BMI (β=0.945, 95% CI; 0.373, 1.517), percent body fat (β=2.527, 95% CI; 1.101, 3.954), and leptin (β=1.857, 95% CI; 0.399, 3.315). Contrary to BPA, PrP concentrations showed consistent inverse associations with BMI (β=-0.588, 95% CI; -1.156, -0.020), percent body fat (β=-1.319, 95% CI; -2.632, -0.005), leptin (β=-1.651, 95% CI; -3.109, -0.193), fasting glucose (β=-1.752, 95% CI; -3.327, -0.177), ln-fasting insulin (β=-0.263, 95% CI; -0.404, -0.122), and ln-HOMA-IR (β=-0.271, 95% CI; -0.418, -0.123) in both single and multi-pollutant models. The urinary ΣDEHPm concentrations showed significant positive associations with adiponectin (β=0.429, 95% CI; 0.090, 0.768) and fasting glucose (β=2.969, 95% CI; 1.158, 4.780). In addition to ΣDEHPm, urinary EtP concentrations also showed a significant positive association with adiponectin (β=0.536, 95% CI; 0.201, 0.872). Urinary MiBP concentrations showed positive associations with ln-fasting insulin (β=0.224, 95% CI; 0.065, 0.382) and ln-HOMA-IR (β=0.239, 95% CI; 0.088, 0.390). Urinary BPS concentrations also showed a positive association with ln-HOMA-IR (β=0.179, 95% CI; 0.032, 0.327). No significant associations with GGT were observed.

Table 2-3. Association of urinary chemicals with obesity and metabolic markers among the study population (n = 459) based on a single-pollutant regression analysis.

Dependent variable	Independent variable	β (95% CI)	*p*-Value
BMI	MMP	-0.691 (-1.264, -0.118)	0.018
	BPA	0.902 (0.331, 1.472)	0.002
	EtP	-0.597 (-1.170, -0.023)	0.041
	PrP	-0.647 (-1.221, -0.073)	0.027
Percent body fat (n = 298)	BPA	2.565 (1.167, 3.962)	<0.001
Adiponectin	MBP	0.431 (0.088, 0.773)	0.014
	MBzP	0.356 (0.017, 0.696)	0.04
	ΣDEHPm	0.471 (0.130, 0.812)	0.007
	EtP	0.569 (0.233, 0.906)	<0.001
	PrP	0.343 (0.003, 0.682)	0.048
Leptin	BPA	1.593 (0.134, 3.051)	0.032
	PrP	-1.717 (-3.176, -0.258)	0.021
Fasting glucose	MBP	2.572 (0.962, 4.182)	0.002
	MiBP	2.807 (1.175, 4.440)	0.001
	MBzP	2.276 (0.680, 3.873)	0.005
	ΣDEHPm	3.775 (2.189, 5.360)	<0.001
Ln-fasting insulin	MiBP	0.192 (0.047, 0.338)	0.01
	BPA	0.150 (0.008, 0.292)	0.04
	PrP	-0.236 (-0.377, -0.095)	0.001
Ln-HOMA-IR	MiBP	0.226 (0.072, 0.379)	0.004
	BPA	0.156 (0.006, 0.306)	0.041
	PrP	-0.254 (-0.403, -0.105)	0.001

Only the urinary chemicals that had significant associations with metabolic markers are shown.

In single-pollutant models, only one chemical is included, and age, urinary nicotine metabolite, and current alcohol consumption were included as covariates.

Unpenalized regression coefficients (β) represent the change in metabolic markers per two-standard deviation increase in ln-transformed creatinine-corrected concentrations of the chemicals.

Table 2-4. Chemicals that were selected via an elastic net regression and their associations with obesity and metabolic markers among the study population (n = 459) based on a multi-pollutant regression analysis.

Dependent variable	Independent variable	β_{EN}	β (95% CI)	*p*-Value
BMI	MMP	-0.227	**-0.685 (-1.257, -0.113)**	**0.019**
	MiBP	0.002	0.351 (-0.237, 0.940)	0.241
	BPA	0.467	**0.945 (0.373, 1.517)**	**0.001**
	EtP	-0.113	-0.542 (-1.107, 0.022)	0.060
	PrP	-0.187	**-0.588 (-1.156, -0.020)**	**0.043**
Percent body fat (n = 298)	MMP	-0.041	-0.868 (-2.156, 0.419)	0.185
	BPA	1.615	**2.527 (1.101, 3.954)**	**0.001**
	BPS	0.180	1.125 (-0.437, 2.688)	0.158
	PrP	-0.507	**-1.319 (-2.632, -0.005)**	**0.049**
Adiponectin	ΣDEHPm	0.070	**0.429 (0.090, 0.768)**	**0.013**
	EtP	0.220	**0.536 (0.201, 0.872)**	**0.002**
Leptin	MBP	-0.050	-1.472 (-2.954, 0.011)	0.052
	BPA	0.340	**1.857 (0.399, 3.315)**	**0.013**
	PrP	-0.447	**-1.651 (-3.109, -0.193)**	**0.027**
Fasting glucose	MBP	0.249	1.125 (-0.695, 2.944)	0.225
	MiBP	0.649	1.079 (-0.778, 2.936)	0.254
	ΣDEHPm	2.171	**2.969 (1.158, 4.780)**	**0.001**
	PrP	-0.311	**-1.752 (-3.327, -0.177)**	**0.029**
Ln-fasting insulin	MMP	0.036	0.111 (-0.033, 0.255)	0.130
	MBP	-0.001	-0.125 (-0.284, 0.033)	0.121
	MiBP	0.142	**0.224 (0.065, 0.382)**	**0.006**
	BPA	0.060	0.112 (-0.031, 0.254)	0.125
	BPS	0.084	0.138 (-0.002, 0.278)	0.054
	PrP	-0.195	**-0.263 (-0.404, -0.122)**	**<0.001**
	BP1	0.040	0.089 (-0.053, 0.231)	0.217
Ln-HOMA-IR	MiBP	0.111	**0.239 (0.088, 0.390)**	**0.002**
	BPS	0.005	**0.179 (0.032, 0.327)**	**0.017**
	PrP	-0.093	**-0.271 (-0.418, -0.123)**	**<0.001**

Both the penalized (elastic net) and unpenalized models adjusted for age, urinary nicotine metabolite, and current alcohol consumption habits.

All chemicals suggested in Table 2-4 were included in both the penalized (elastic net) models.

Chemicals selected via elastic net regression were included in the OLS models.

Elastic net regression coefficients (β_{EN}) with 10-fold cross validations represent the change in metabolic markers per two-standard deviation increase in ln-transformed creatinine-corrected concentrations of chemicals.

Unpenalized regression coefficients (β) represent the change in metabolic markers per two standard deviation increase in ln-transformed creatinine-corrected concentrations of chemicals.

p-Values were calculated from the results of unpenalized regression.

Bold numbers indicate statistical significance ($p<0.05$).

Characteristics of independent variables shown in bold font represent statistical significance at the $p<0.05$ level.

2.4 Discussion

In the current study, significant positive associations of several phthalate metabolites with fasting glucose and HOMA-IR were found in both single- and multi-pollutant models. Though adiponectin has been reported to have important roles in mediating T2DM and insulin resistance, only limited studies exist on the associations between chemicals and T2DM-related markers. Thus, our findings on significant positive associations of ΣDEHPm and EtP with serum adiponectin are important, and mechanisms of involvement of adiponectin of developing insulin resistance need to be explored.

Significant positive associations of ΣDEHPm and EtP with serum adiponectin level were consistently observed in both single- and multi-pollutant models. The positive association of urinary EtP with adiponectin was observed for the first time in human population. The associations between phthalates and adiponectin have been assessed in a few epidemiological studies, and most studies were focused on human infants and the associations were inconsistent. A Canadian birth cohort study reported no significant associations of prenatal exposure to phthalate metabolites including MEP, MBP, MBzP, MCPP, ΣDEHPm with adiponectin in infants (n = 1,080), while among male infants (n = 578), the third quartile of MCPP showed significantly reduced odds of low adiponectin (Ashley-Martin et al., 2014). Another Japanese birth cohort study (n = 365) also reported no significant associations of maternal serum phthalate metabolite levels with cord blood adiponectin levels (Minatoya et al., 2018). In contrast, in a Chinese case-only study of patients with diabetes (n = 329, aged 29 to 93 years), positive associations were observed between adiponectin and phthalate metabolites including secondary DEHP metabolites, MBzP, MCPP, MBP, and MiBP (Duan et al., 2017), which are similar with the present study. In the present study, significant positive associations of MBP and MBzP with serum adiponectin level were observed in single-

pollutant model (Table 2-3). The reason for inconsistent associations of phthalate metabolites with adiponectin from other birth cohort studies might be due to difference of age among the population. Similar observation reported from the Chinese case-only study (Duan et al., 2017) supports the importance of age: the positive associations of several phthalate metabolites with adiponectin among adult populations, not in infants. Meanwhile, no previous epidemiological study has reported an association between EtP and serum adipokines, and therefore, I could not directly compare our observations with those of different study populations. Because there were relatively higher levels of urinary EtP in the Korean population compared to the levels of other countries (Asimakopoulos et al., 2014b; de Renzy-Martin et al., 2014; Kang et al., 2016; Larsson et al., 2014), further studies are needed to confirm this observation in other populations. One possible explanation of the positive associations of several phthalate metabolites and EtP with adiponectin level is that chemicals act as PPARγ agonists. PPARγ agonistic activities of several phthalates and parabens have been reported previously (Zhang et al., 2017; Pereira-Fernandes et al., 2013), and serum adiponectin is considered to be a marker of PPARγ activation (Yang et al., 2004; Kusminski et al., 2009). However, because present study is cross-sectional, the associations and underlying mechanisms need to be further investigated and confirmed in other experimental and epidemiological studies.

The positive associations between several phthalates with fasting glucose and HOMA-IR observed in the present study population (Tables 2-3 and 2-4) are in line with by several epidemiological observations based on national biomonitoring programs. For example, Song et al. reported an association between higher urinary concentrations of phthalates and BPA and elevated T2DM risk in a meta-analysis (Song et al., 2016). In the Canadian Health Measures Study (CHMS, n = 2,119), higher urinary DEHP metabolites were associated with increased fasting glucose (Dales et al., 2018). In NHANES participants without diabetes (n = 3,083, age = 12-80 years), urinary MBP, MiBP, MCPP and ΣDEHPm levels were positively

associated with increased fasting glucose and HOMA-IR (Huang et al., 2014). Similarly, among the adolescents from the NHANES 2009-2012 survey (n = 356, age = 12-18 years), the sum of the DEHP metabolites in the urine showed positive associations with HOMA-IR (Attina and Trasande, 2015). However, contrasting evidence has also been reported by many other studies (James-Todd et al., 2016; Shapiro et al., 2015). In a pregnancy cohort, the highest quartile of the sum of the DEHP metabolites in the urine showed significantly reduced odds of having impaired glucose tolerance in the 2nd trimester, and higher 2nd trimester MEP concentrations were associated with a higher risk of impaired glucose tolerance (James-Toddet al., 2016). Similarly, among a reasonably sized population of pregnant Canadian women (n = 1,274), no significant associations were observed between impaired glucose tolerance and some phthalates and BPA measured in the first trimester (Shapiro et al., 2015). Inconsistent observations between these studies may be due to differences in the population characteristics of each study such as age, sex, race, ethnicity, BMI, and pregnancy status. It may be due to the cross-sectional study design that could often lead to chance observations. Thus, these inconsistencies should be examined in longitudinal studies that incorporate these various characteristics in their study participants.

Significant associations that were consistently detected between ΣDEHPm and fasting glucose in both the single- and multi-pollutant models (Tables 2-3 and 2-4) can be partly explained by experimental evidence. Several mechanisms, which support the positive associations of DEHP with fasting glucose, have been suggested through experimental studies. In rat, exposure to DEHP induced liver damage, glucose tolerance, and insulin tolerance along with reduced expression of the insulin receptor and glucose transporter 4 (GLUT4) proteins, which are responsible for glucose transport (Zhang et al., 2017). In the same study, authors observe that DEHP could lead to the activation of PPARγ and induce oxidative stress in L02 cells, a human hepatocyte line (Zhang et al., 2017). These

observations in the L02 cells and the rat models suggest that DEHP may cause PPARγ-mediated hepatotoxicity and could be linked to glucose tolerance and insulin resistance. Moreover, in a rat skeletal muscle cell model of cultured L6 myoblast cells, MEHP exposure was shown to have effects on insulin signaling and GLUT4 translocation (Viswanathan et al., 2017). In addition, another evidence supports that DEHP can impair pancreatic β-cell function and whole body glucose homeostasis in rats (Lin et al., 2011).

The significant negative association between PrP and fasting glucose and HOMA-IR is interesting, but there is not an easy, clear explanation for this observation. Considering the cross-sectional nature of the present study, studies should be conducted to further validate these observations in other populations and confirm the underlying mechanism, if any, in experimental studies.

It should be noted that the urinary chemicals that were measured from the one-spot urine samples in the present study have short half-lives in the body (Abbas et al., 2010; Koch et al., 2006; Thayer et al., 2015), and therefore this study could not provide reliable inference for the long-term exposure to them. However, the present study is unique in that it considered multiple urinary chemicals, i.e., phthalate metabolites, bisphenols, parabens, TCS, and benzophenones, in relation to metabolic markers among premenopausal adult females. In addition, I chose the chemicals via elastic net regression and conducted multi-pollutant models to identify possible chemical determinants of the given outcome. Moreover, this observation was tested again with secondary analysis with the chemicals that were found to be significantly correlated to each other.

The results of the present study emphasize the importance of phthalate exposure on T2DM-related markers, as well as the possible involvement of serum adipokines. In addition, the association between EtP exposure and serum adiponectin levels observed in this

population of adult females suggests the potential involvement of EtP in lipid metabolism and outlines the importance of follow-up epidemiological and experimental studies.

Chapter 3 Association of blood heavy metals with obesity and metabolic markers

3.1 Introduction

Heavy metals exist ubiquitously and are persistent in the environment (Duruibe et al., 2007; Järup, 2003; Pacyna et al., 2007). The anthropogenic sources of heavy metals include releases from usage of heavy metals impurities, such as coal-fired power and heat production, intentional extraction and use of heavy metals, such as heavy metal mining and releases from waste incineration (Duruibe et al., 2007; Järup, 2003; Pacyna et al., 2007). Humans are exposed to heavy metals owing to their ubiquitous existence. Exposure to heavy metals occurs through the environment, including drinking water, food, air, soil, and dust (Järup, 2003; Pacyna et al., 2007).

Heavy metals such mercury (Hg), cadmium (Cd), and lead (Pb) have been considered as endocrine disrupting chemicals due to their reproductive and neurodevelopmental toxicity (Gorini et al., 2014; Wirth and Mijal, 2010). Many studies have reported associations of heavy metals, such as Hg, Cd, and Pb, with obesity, but evidence is inconsistent (Cho et al., 2014; Fan et al., 2017; Gambelunghe et al., 2016; Lee et al., 2016; Noor et al., 2018; Rhee et al., 2013; Riederer et al., 2013; Rothenberg et al., 2015; Rotter et al., 2015; Shin et al., 2018; Skalnaya et al., 2014; Son et al., 2015). Heavy metals are also reported to disrupt metabolic markers such as fasting glucose and insulin (Chen et al., 2009; González-Villava et al., 2016; Tinkov et al., 2015; Tinkov et al., 2017). Associations with insulin resistance (IR) or diabetes mellitus (DM) reported in epidemiological studies have also been supported by experimental studies (Chen et al., 2009; González-Villava et al., 2016; Tinkov et al., 2015; Tinkov et al.,

2017). Several *in vivo* studies have reported that exposure to heavy metals can induce hyperglycemia or IR (Ibrahim et al., 2012; Novakova et al., 2015). Oxidative stress has been suggested as a possible mechanism for heavy metal induced IR and DM (Chen et al., 2009; González-Villava et al., 2016; Tinkov et al., 2015; Tinkov et al., 2017).

Despite experimental evidences, the associations of heavy metals with IR or DM are not consistent in epidemiological studies (Borné et al., 2014; Chang et al., 2011; He et al., 2013; Jeppesen et al., 2015; Nie et al., 2016; Swaddiwudhipong et al., 2012). These inconsistent associations may be attributed to differences in characteristics of study populations, outcome variables, and statistical analyses. The fact that several metabolic markers are closely associated with one another may, in part, explain these inconsistent observations (Wu et al., 2011). A marker can be on a causal pathway between chemical exposure and other markers, and therefore the relationship between the markers should be well understood. However, only a few studies have considered such relationship for association studies between chemical exposure and metabolic markers. Significant indirect effects of oxidative stress markers such as malondialdehyde (MDA) and γ-glutamiltransferase (GGT) have been reported in the association between phthalate exposure and type 2 diabetes mellitus (T2DM) or IR related markers based on mediation analysis (Dong et al., 2018; Li et al., 2019). Similar approach was rarely been made on association studies between metal exposure and IR-related markers such as adipokines.

In the present study, I determined the blood concentrations of the heavy metals, i.e., Hg, Pb, and Cd, in women of reproductive age, were determined and associated with serum adiponectin, leptin, GGT, fasting glucose, and homeostasis model assessment-insulin resistance (HOMA-IR). Mediation analysis was subsequently performed to understand how certain markers could influence the association between heavy metal exposure and IR. The results of this study will help better understand the effects of heavy metal exposure on

metabolic markers among adult women of reproductive age, and to identify potential mediators of such association.

3.2 Materials and methods

3.2.1 Study population, questionnaire, and sample collection

The participating women (n = 516) between 20 and 48 years of age were recruited from five university hospitals or public health center, located in Seoul, Incheon, Ansan, and Jeju in Korea, between 2015 and 2016. The spot blood and urine samples were collected from the participants after at least eight hours of fast\. Serum was separated from whole blood by centrifugation for 15 min at 3000 rpm in serum separator tube (SST). The separated serum and EDTA-treated blood samples were stored in cryovials at -80°C before analysis. The urine samples were stored in tubes at -40°C before analysis. Participants had completed a questionnaire at the time of recruitment.

Among the women initially recruited, pregnant women (n=33) and participants with fasting glucose ≥126 mg/dL (n=2) were excluded from the study. In addition, participants with insufficient amount of blood samples, and with missing demographic and behavioral information were also excluded. The final number of subjects in the study was 456. This study was approved by the Institutional Review Boards of School of Public Health, Seoul National University, and all participating university hospitals (IRB No. 1509/001-011). Informed consent was obtained from the participants.

3.2.2 Measurement of in blood and urine samples

Urinary nicotine metabolite, along with serum glucose, insulin, and GGT were estimated by a commercial laboratory (Green Cross LabCell, Yongin, Korea). Adiponectin and leptin in serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Duoset, R&D Systems, Minneapolis, MN, USA). IR status was estimated using the HOMA-IR as follows: fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5 (Matthews et al., 1985).

Blood samples were mixed thoroughly in a roll-mixer for 1 hour before analysis and did not require any pretreatment. Total Hg levels in blood samples were measured following US EPA method 7473 (2007) using an automatic Hg analyzer (SP-3D, Nippon Instruments Co., Japan), which incorporated heat vaporization, gold amalgamation, and cold vapor atomic absorption technique. Pb and Cd in blood samples were analyzed using atomic absorption spectrophotometer (AAS; GFAAS; Z-5700, Hitachi, Japan) with graphite furnace (A-type graphite cuvette, Hitachi, Japan) at the wavelength of 283.3 nm and 228.8 nm, respectively. For Pb and Cd analysis, 100 μL sample of blood was diluted with 1 % Triton X-100 and 1% ammonium dihydrogen phosphate ($NH_4H_2PO_4$) as a modifier. The limits of detection (LODs) for blood Hg, Pb, and Cd were 0.2 μg/L, 0.16 μg/dL, and 0.22 μg/L, respectively. The precision and accuracy of the analysis were tested using Certified Reference Materials (CRM), and external quality control was achieved according to standard reference material 56 of German External Quality Assessment Scheme (G-EQUAS).

3.2.3 Statistical analysis

Proxy value (LOD divided by square root of 2) was used to replace the non-detects. Spearman's correlations were performed to determine associations between exposure to each metal and metabolic markers. Linear regression analyses were performed for associations between metals and adiponectin, leptin, and GGT. Due to right skewness, GGT, insulin, and HOMA-IR were ln-transformed. In crude model, no covariates were adjusted. In adjusted models, covariates were chosen based on a previous study, which reported possible risk factors for T2DM (Zheng et al., 2018). In the adjusted models of both linear and logistic regression, age (categorical: 20-29, 30-39, 40-48 years), urinary nicotine metabolite (categorical: <10, 11-499, ≥500 ng/mL), and alcohol consumption at the time of study (categorical: drinker and non-drinker) were included as covariates. Further, alcohol consumption is known to influence GGT, so we confirmed whether the associations between blood metal and serum GGT were independent of alcohol consumption at the time of study by either adding in or removing from the adjusted model. Independent variables, i.e., blood heavy metal concentrations, were divided into quartiles.

Logistic regression was also performed to look for effects of outcome variables with cut-off criteria. Logistic regression models were used to calculate adjusted odds ratios (aORs). Criteria of cut-off points were based on previous studies. Overweight and obesity was defined based on the WHO's risk cut points for Asian population, i.e., BMI above 23 kg/m^2, (World Health Organization, 2000). Impaired fasting glucose (IFG) was defined as glucose ≥100 mg/dL (American Diabetes Association, 2015). Because cut-off point of HOMA-IR ≥75^{th} is associated with metabolic syndrome-related markers (Tang et al., 2015), we chose 75^{th} as the cut-off point for HOMA-IR. Heavy metal concentrations, along with the covariates, were also divided into quartiles in the logistic regression analysis.

Mediation analysis was performed using bootstrapping; and model 4 for one mediator and model 6 for serial two-mediators were performed with macro PROCESS (V3.3) (Hayes et al., 2017) using same covariates as used in linear and logistic regression analysis. A value of $p<0.05$ was considered as statistically significant. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3.3 Results

3.3.1 Characteristics of study population

Participating women were aged between 20 and 48 years, with about a half of the women (48.2%) in their thirties (Table 3-1). Most participants (63.6%) showed normal range of BMI (18.5-22.9 kg/m^2), and 6.4% and 3.3% of the participants were underweight (<18.5 kg/m^2) and moderately obese (≥30 kg/m^2), respectively.

Table 3-1. Characteristics of the study population (n = 456).

	N (%)
Age (years)	
20-29	126 (27.6)
30-39	220 (48.2)
40-48	110 (24.1)
Body mass index (kg/m^2)	
<18.5	29 (6.4)
18.5-22.9	290 (63.6)
23.0-24.9	66 (14.5)
25.0-29.9	56 (12.3)
≥30.0	15 (3.3)
Urinary nicotine metabolite (ng/mL)	
<10	406 (89.0)
11-499	24 (5.3)
≥500	26 (5.7)
Current alcohol drinking	
Non-drinker	136 (29.8)
Drinker	320 (70.2)
	median (25th-75th)
Adiponectin (μg/mL)	6.6 (5.4, 7.8)
Leptin (ng/mL)	9.7 (5.9, 15.5)
GGT (U/L)	12.0 (10.0, 17.0)
Fasting glucose (mg/dL)	88.0 (83.0, 93.0)
Fasting insulin (μU/mL)	6.1 (4.0, 8.6)
HOMA-IR	1.3 (0.9, 1.9)

3.3.2 Blood metal concentrations, parameters, and their correlations

Detection frequencies and concentrations of heavy metals in blood samples are shown in Table 3-2. The median concentrations of blood Hg, Cd, and Pb with interquartile range were 2.6 (1.8, 3.7) μg/L, 1.0 (0.7, 1.4) μg/L, and 1.2 (0.9, 1.6) μg/dL, respectively. Blood Pb showed weak correlation with Hg (ρ = 0.156, p = 0.001) and Cd (ρ = 0183, p < 0.001), but Hg and Cd did not show significant correlation with each other (Fig. 3-1). Parameters related to obesity and IR showed weak to high correlations (Fig. 3-1). BMI, a surrogate marker of obesity, particularly, showed significant correlation with other metabolic markers measured in this study.

Table 3-2. Detection frequency and levels of heavy metals in the blood of participating women (n = 456).

	Detection frequency (%)	**Mean ± SD**	**Median (25^{th}-75^{th})**
Hg (µg/L)	100	3.0±1.9	2.6 (1.8-3.7)
Cd (µg/L)	99.1	1.1±0.6	1.0 (0.7-1.4)
Pb (µg/dL)	99.6	1.3±0.8	1.2 (0.9-1.6)

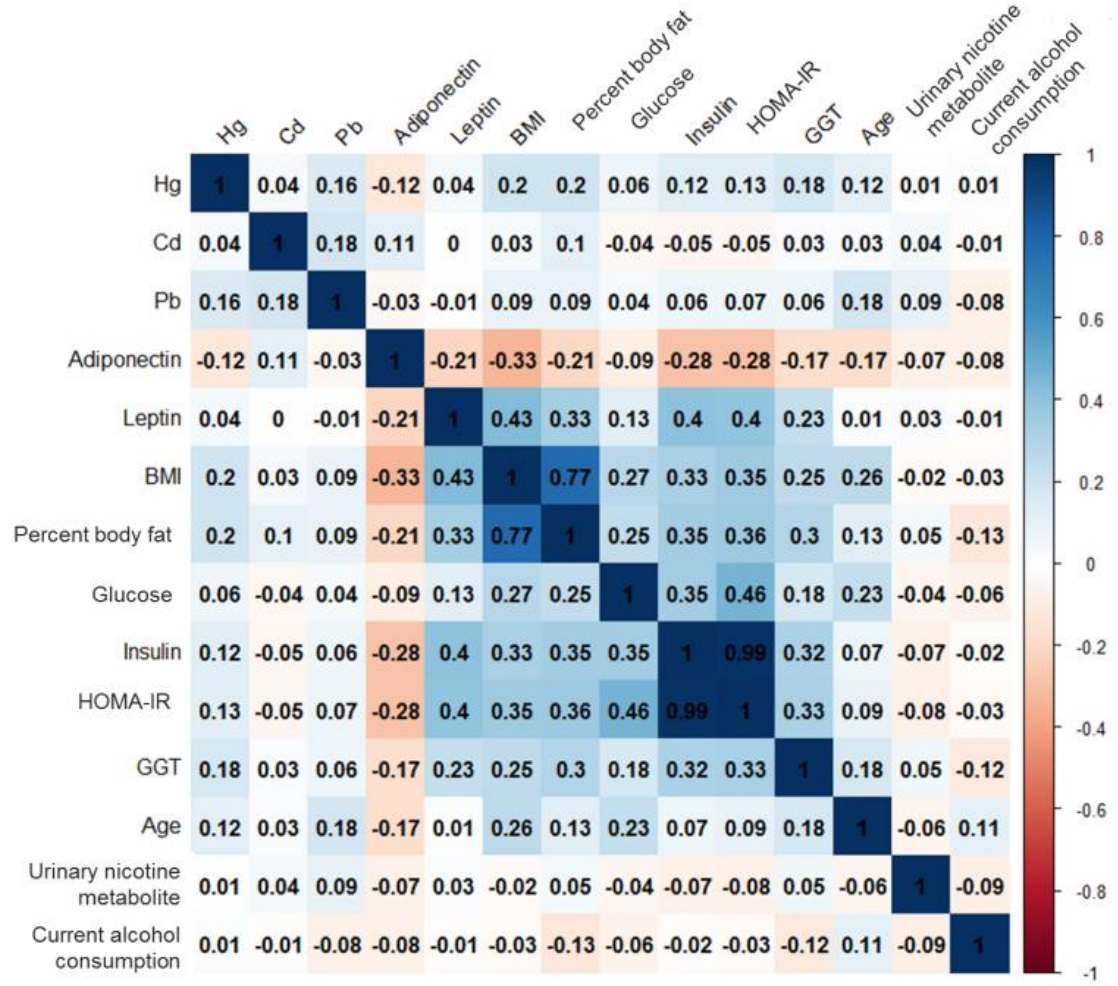

Fig. 3-1. Results of Spearman's correlations (n = 456; except for percent body fat (n = 294). All categorical variables (urinary nicotine metabolite and current alcohol consumption) were coded as increasing numbers as frequency or value increase.

3.3.3 Associations of heavy metals with BMI, adipokines, GGT, fasting glucose, and HOMA-IR

Higher blood mercury concentrations were associated with higher BMI (≥23 kg/m^2) (Fig. 3-2). The increased adjusted odds ratio (aOR) for BMI ≥23 kg/m^2 was found in the third and fourth quartiles of blood Hg concentrations. The aOR for BMI ≥23 kg/m^2 per quartile increase of blood Hg was 1.396 (95% CI: 1.156, 1.686). The increased aOR for BMI ≥23 kg/m^2 was found only in the second quartile of blood Cd concentrations, and aOR per quartile of blood Cd was 1.093 (95% CI: 0.909, 1.315). Blood Pb concentrations did not show associations with BMI (95% CI included one). In secondary analysis, percent body fat was used as an obesity marker instead of BMI, a surrogate marker of obesity. Though, due to small sample size of percent body fat, BMI was used in main statistical analyses, association of blood heavy metal concentrations with obesity using percent body fat was also found. In accordance with BMI, blood Hg and Cd concentrations showed significant positive associations with percent body fat (Table 3-3).

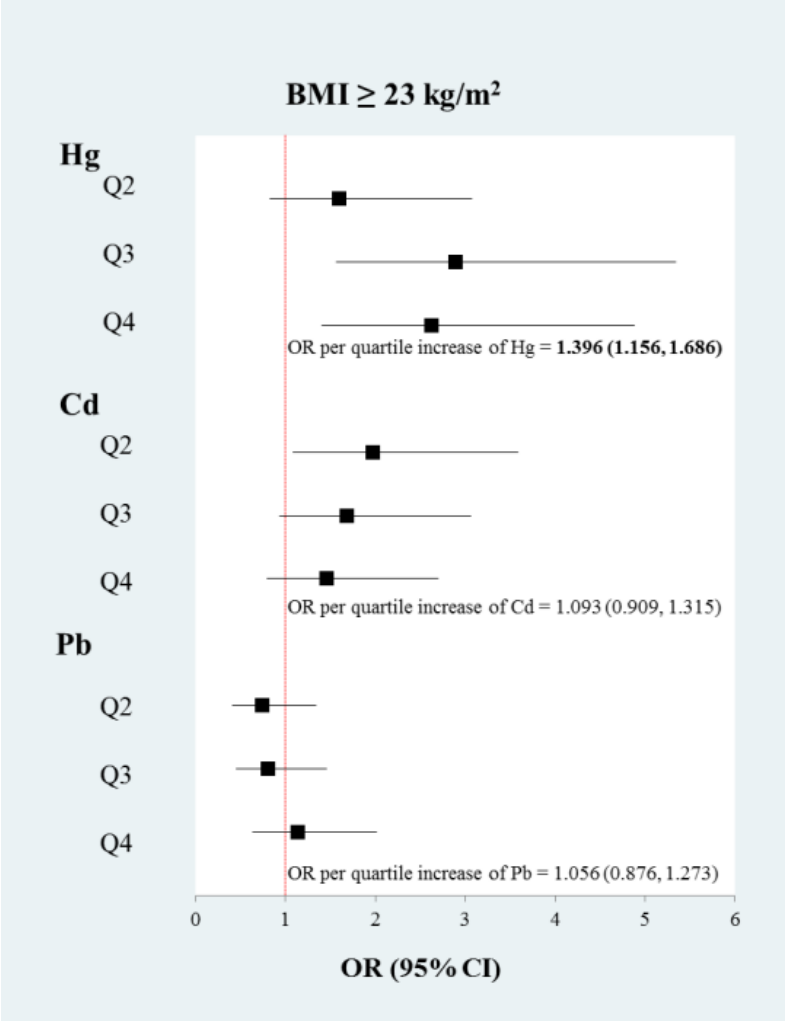

Fig. 3-2. Association of heavy metals with BMI (≥23 kg/m^2) as the outcome (n = 456). The Model was adjusted for age, urinary nicotine metabolite, and current alcohol drinking.

Table 3-3. Association of heavy metals with percent body fat as the outcome (n = 294).

	Crude[a]		**Adjusted**[b]	
	β (95% CI)	***p*-Value**	**β (95% CI)**	***p*-Value**
Percent body fat				
Hg				
Q1	Ref		Ref	
Q2	0.595 (-1.456, 2.645)	0.568	0.753 (-1.314, 2.821)	0.474
Q3	**2.452 (0.547, 4.356)**	**0.012**	**2.396 (0.483, 4.309)**	**0.014**
Q4	**2.764 (0.803, 4.726)**	**0.006**	**2.807 (0.831, 4.782)**	**0.006**
Per quartile increase	**1.016 (0.396, 1.635)**	**0.001**	**1.006 (0.382, 1.631)**	**0.002**
Cd				
Q1	Ref		Ref	
Q2	1.510 (-0.227, 3.246)	0.088	1.506 (-0.246, 3.258)	0.092
Q3	1.790 (-0.172, 3.753)	0.074	1.984 (-0.060, 4.028)	0.057
Q4	**2.187 (0.042, 4.333)**	**0.046**	2.039 (-0.190, 4.267)	0.073
Per quartile increase	**0.743 (0.087, 1.399)**	**0.027**	**0.730 (0.040, 1.420)**	**0.038**
Pb				
Q1	Ref		Ref	
Q2	0.014 (-1.988, 2.017)	0.989	-0.149 (-2.169, 1.872)	0.885
Q3	0.422 (-1.580, 2.425)	0.678	0.234 (-1.832, 2.301)	0.824
Q4	0.944 (-1.065, 2.953)	0.356	0.562 (-1.509, 2.634)	0.594
Per quartile increase	0.326 (-0.305, 0.958)	0.310	0.212 (-0.443, 0.867)	0.525

Bold number indicates statistical significance ($p<0.05$).

[a]Crude model : no covariates were adjusted.

[b]Adjusted model : adjusted for age, urinary nicotine metabolite, and current alcohol drinking.

Table 3-4 shows associations of blood metal concentrations with adiponectin and leptin. Higher blood Hg concentrations were significantly associated with lower serum adiponectin levels in both crude (β = -0.201 per quartile increase of Hg; 95% CI: -0.356, -0.046) and adjusted (β = -0.179 per quartile increase of Hg; 95% CI: -0.333, -0.024) models. Serum adiponectin was marginally significantly (p<0.1) reduced in the third and fourth quartiles of blood Hg in both crude and adjusted models. In contrast, higher blood Cd concentrations were associated with higher serum adiponectin in both crude (β = 0.202 per quartile increase of Cd; 95% CI: 0.048, 0.356) and adjusted (β = 0.206 per quartile increase of Cd; 95% CI: 0.052, 0.360) models. Serum adiponectin was significantly reduced in the fourth quartile of blood Cd in both crude and adjusted models. Serum leptin did not show any statistically significant association with blood heavy metal concentrations.

Table 3-4. Association of heavy metals with adipokines as the outcome (n = 456).

	Crude[a]		**Adjusted**[b]	
	β (95% CI)	***p*-Value**	**β (95% CI)**	***p*-Value**
Adiponectin				
Hg				
Q1	Ref		Ref	
Q2	0.076 (-0.418, 0.569)	0.764	0.025 (-0.464, 0.514)	0.920
Q3	-0.484 (-0.969, 0.001)	0.051	-0.417 (-0.899, 0.065)	0.090
Q4	-0.480 (-0.970, 0.010)	0.055	-0.449 (-0.936, 0.038)	0.071
Per quartile increase	**-0.201 (-0.356, -0.046)**	**0.011**	**-0.179 (-0.333, -0.024)**	**0.023**
Cd				
Q1	Ref		Ref	
Q2	-0.158 (-0.651, 0.336)	0.530	-0.077 (-0.566, 0.411)	0.756
Q3	0.279 (-0.203, 0.762)	0.256	0.387 (-0.093, 0.868)	0.114
Q4	**0.531 (0.043, 1.018)**	**0.033**	**0.535 (0.047, 1.023)**	**0.032**
Per quartile increase	**0.202 (0.048, 0.356)**	**0.011**	**0.206 (0.052, 0.360)**	**0.009**
Pb				
Q1	Ref		Ref	
Q2	-0.058 (-0.551, 0.436)	0.818	0.029 (-0.461, 0.520)	0.907
Q3	-0.484 (-0.975, 0.008)	0.054	-0.287 (-0.785, 0.210)	0.257
Q4	-0.111 (-0.605, 0.384)	0.660	0.028 (-0.470, 0.526)	0.912
Per quartile increase	-0.076 (-0.232, 0.080)	0.340	-0.021 (-0.179, 0.137)	0.793
Leptin				
Hg				
Q1	Ref		Ref	
Q2	1.184 (-0.915, 3.282)	0.268	1.180 (-0.938, 3.297)	0.274
Q3	1.167 (-0.896, 3.230)	0.267	1.131 (-0.955, 3.217)	0.287
Q4	0.783 (-1.301, 2.868)	0.461	0.748 (-1.362, 2.858)	0.486
Per quartile increase	0.234 (-0.424, 0.892)	0.486	0.220 (-0.447, 0.888)	0.517
Cd				
Q1	Ref		Ref	
Q2	0.542 (-1.560, 2.643)	0.613	0.537 (-1.585, 2.659)	0.619
Q3	0.796 (-1.260, 2.852)	0.447	0.738 (-1.349, 2.825)	0.488
Q4	0.308 (-1.770, 2.385)	0.771	0.195 (-1.924, 2.313)	0.857
Per quartile increase	0.120 (-0.536, 0.776)	0.719	0.083 (-0.586, 0.752)	0.808
Pb				
Q1	Ref		Ref	
Q2	0.169 (-1.924, 2.261)	0.874	0.190 (-1.927, 2.307)	0.860
Q3	0.595 (-1.488, 2.679)	0.575	0.595 (-1.552, 2.743)	0.586
Q4	0.021 (-2.076, 2.118)	0.985	0.053 (-2.096, 2.202)	0.961
Per quartile increase	0.049 (-0.612, 0.709)	0.885	0.052 (-0.629, 0.733)	0.881

Bold number indicates statistical significance ($p<0.05$).
[a]Crude model: no covariates were adjusted.
[b]Adjusted model: adjusted for age, urinary nicotine metabolite, and current alcohol drinking.

Blood Hg levels showed significant association with ln-GGT (Table 3-5). Higher blood Hg concentrations were significantly associated with increased ln-GGT in both crude (β = 0.078 per quartile increase of Hg; 95% CI: 0.036, 0.121), and adjusted models (β = 0.070 per quartile increase of Hg; 95% CI: 0.029, 0.112). GGT is known to be a marker of excessive alcohol consumption (Teschke et al., 1997). The association between blood Hg and GGT shown in the present population was independent of alcohol consumption at the time of study (data not shown).

Table 3-5. Association of heavy metals with GGT as the outcome (n = 456).

	Crude[a]		**Adjusted 1**[b]		**Adjusted 2**[c]	
	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value
Ln-GGT[d]						
Hg						
Q1	Ref		Ref		Ref	
Q2	0.117 (-0.018, 0.251)	0.088	0.120 (-0.014, 0.254)	0.080	0.119 (-0.014, 0.251)	0.079
Q3	**0.160 (0.028, 0.292)**	**0.018**	**0.139 (0.006, 0.271)**	**0.040**	**0.138 (0.007, 0.269)**	**0.039**
Q4	**0.247 (0.114, 0.381)**	**<0.001**	**0.229 (0.095, 0.363)**	**0.001**	**0.228 (0.096, 0.360)**	**0.001**
Per quartile increase	**0.078 (0.036, 0.121)**	**<0.001**	**0.071 (0.028, 0.113)**	**0.001**	**0.070 (0.029, 0.112)**	**0.001**
Cd						
Q1	Ref		Ref		Ref	
Q2	0.093 (-0.043, 0.229)	0.180	0.079 (-0.057, 0.215)	0.254	0.079 (-0.055, 0.214)	0.247
Q3	0.047 (-0.087, 0.180)	0.493	0.018 (-0.115, 0.152)	0.788	0.030 (-0.102, 0.163)	0.651
Q4	0.057 (-0.078, 0.191)	0.410	0.041 (-0.094, 0.177)	0.551	0.036 (-0.098, 0.170)	0.600
Per quartile increase	0.012 (-0.030, 0.055)	0.564	0.007 (-0.036, 0.049)	0.766	0.006 (-0.036, 0.049)	0.775
Pb						
Q1	Ref		Ref		Ref	
Q2	0.052 (-0.084, 0.187)	0.454	0.045 (-0.091, 0.180)	0.516	0.046 (-0.088, 0.180)	0.497
Q3	0.048 (-0.087, 0.182)	0.488	0.008 (-0.130, 0.145)	0.914	0.003 (-0.133, 0.139)	0.964
Q4	0.127 (-0.009, 0.262)	0.067	0.102 (-0.035, 0.238)	0.146	0.084 (-0.052, 0.220)	0.223
Per quartile increase	0.038 (-0.005, 0.080)	0.085	0.027 (-0.016, 0.071)	0.220	0.021 (-0.022, 0.064)	0.333

Bold number indicates statistical significance ($p<0.05$).
[a]Crude model: no covariates were adjusted.
[b]Adjusted model: adjusted for age, and urinary nicotine metabolite.
[c]Adjusted model: adjusted for age, current alcohol consumption, and urinary nicotine metabolite.

Heavy metals did not show significant association with fasting glucose levels (Fig. 3-3A). The aOR for HOMA-IR $\geq 75^{th}$ per quartile increase of blood Hg concentrations was 1.341 (95% CI: 1.100, 1.634) (Fig. 3-3B). Significant increase of aOR for HOMA-IR $\geq 75^{th}$ in the fourth quartile of blood Hg, in the second quartile of blood Cd, and in the third and fourth quartiles of blood Pb was found.

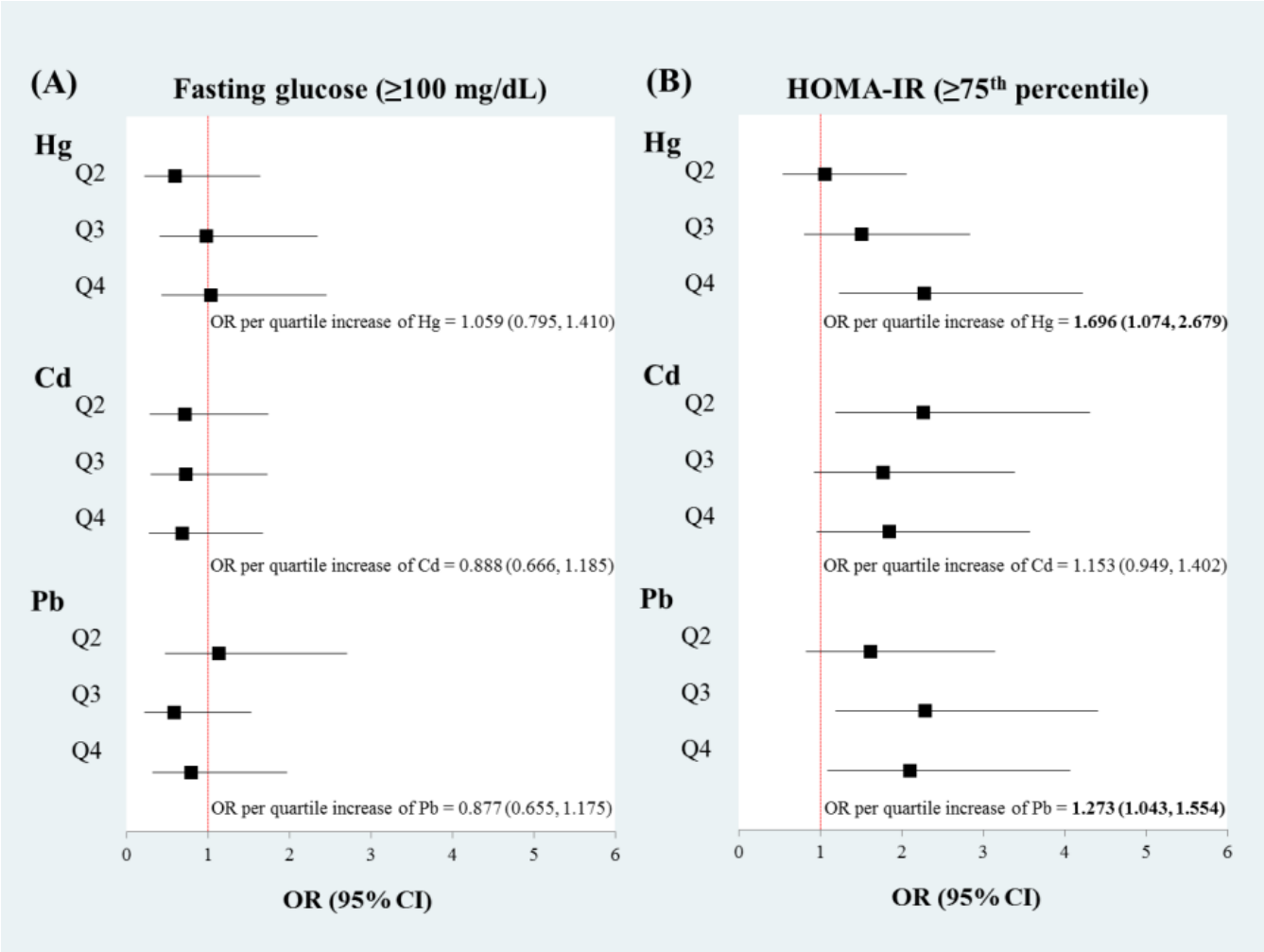

Fig. 3-3. Association of heavy metals with (A) fasting glucose (≥100 mg/dL) and (B) HOMA-IR (≥75th percentile) as the outcome (n = 456). The models were adjusted for age, urinary nicotine metabolite, and current alcohol drinking.

3.3.4. Mediation of adiponectin, and GGT in the relationship between heavy metals and HOMA-IR

Higher blood Hg level was associated with higher BMI and GGT, and lower adiponectin (Fig. 3-1 and Tables 3-4 and 3-5). According to the mediation analysis, adiponectin, GGT, and BMI were found to be significant mediators of the association between Hg and HOMA-IR, with an estimated mediation range of 18-34% in the adjusted model (Table 3-6).

Table 3-6. Indirect, total indirect, and direct effects of Hg on HOMA-IR (n = 456).

	Crude[a]			Adjusted[b]		
	Direct effect (95% CI)	Indirect effect (95% CI)	Estimated percent mediated (%)	Direct effect (95% CI)	Indirect effect (95% CI)	Estimated percent mediated (%)
Adiponectin	0.257 (0.055, 0.459)	0.066 (0.015, 0.135)	20	0.263 (0.060, 0.466)	0.059 (0.007, 0.125)	18
Ln-GGT	0.215 (0.009, 0.421)	0.107 (0.048, 0.191)	33	0.220 (0.013, 0.427)	0.103 (0.044, 0.189)	32

X (Hg) is an ordinal (categorical) variable consisted of four quartiles.

Y (HOMA-IR≥75th percentile) is a dichotomous variable.

All mediators (adiponectin, ln-GGT, and BMI) are continuous variables.

[a]No covariates were adjusted.

[b]Age, urinary nicotine metabolite, and current alcohol drinking were used as covariates.

3.4 Discussion

3.4.1. Association of heavy metals with and obesity and insulin resistance

Significant positive association of blood Hg level with a surrogate marker of obesity (BMI) (Fig. 3-2) is comparable to the observations from Korean National Health and Nutrition Examination (Cho et al., 2014; Lee et al., 2016; Shin et al., 2018) with large sample size (n=1567 - 9228). Similarly, higher hair Hg levels were associated with BMI in young adults of Russia (35 years old or younger, Skalnaya et al., 2014). However, inconsistent results have been reported in the US populations. Inverse associations with BMI was reported in the US adults from the National Health and Nutrition Examination Survey (NHANES) (n = 962) (Rothenberg et al., 2015), and no associations were found in children and adolescents (n = 5404) from NHANES (Fan et al., 2017). The difference in blood Hg levels among countries may partly explain the observed inconsistency. Median blood Hg concentration among Korean population is 3.7 μg/L (Lee et al., 2016), which is 2.5 folds higher than those reported in the US population (1.5 μg/L) (Rothenberg et al., 2015). However, other demographic and/or lifestyle characteristics may influence the occurrence of obesity, independent of Hg.

Inverse or no associations between Cd and/or Pb exposure and obesity have been reported. The current study also presents similar results. At the current levels of exposure, Cd or Pb may not be associated with increased risk of obesity among Korean women. In Chinese healthy women aged between 16 and 35 years (n = 4400), BMI has been shown to be inversely associated with both blood Cd and Pb concentrations (Liu et al., 2013). Similarly, no significant associations between hair Cd and Pb content, and BMI have been observed in adults aged between 22 and 60 years (n = 1229) (Skalnaya et al., 2014). Similar observations

have also been reported in other cross-sectional studies (Noor et al., 2018; Rhee et al., 2013; Son et al., 2015).

Overall, increased ORs of high HOMA-IR were found in Hg, Cd, and Pb (Fig. 3-3). Positive associations of blood Hg and Pb with IR in the present female population (Fig. 3-3) are similar to several previous epidemiological studies, which have reported positive associations with IR-related markers or DM. Hg exposure measured in toenail was positively associated with incidence of DM in a cohort study of an 18-year follow-up of the US young adults aged between 20 and 32 years at enrollment (n = 3875) (He et al., 2013). The positive association has also been observed in Korean population (Kim et al., 2015). During Korea NHANES (KNHANES) study, from 2008 to 2010, blood Hg levels showed a significant positive association with HOMA-IR in non-diabetic Korean population of both sexes (Kim et al., 2015). High risk of DM incidence of the highest Cd exposure category compared to the lowest category has been reported in a meta-analysis (Tinkov et al., 2017). Blood Pb levels and fasting glucose levels are higher among industrial workers than among their non-industrial counterparts in the United Arab Emirates (Bener et al., 2001). However, contradictory reports also exist in literature (Forte et al., 2013; Lee and Kim, 2013; Moon, 2014). The discrepancies might be due to different characteristics of study population, and outcome variables. The population in the current study was healthy without DM, which could not show relationship with DM.

Although the underlying mechanism of exposure to heavy metals and IR has not yet been completely understood, oxidative stress process is a possible mechanism (Chen et al., 2009; Gonzalez-Villalva et al., 2016; Houstis et al., 2006; Tangvarasittichai, 2015; Tinkov et al., 2015;).

3.4.2. Association of heavy metals with adipokines and GGT

Significant associations between blood Hg or Cd and serum adiponectin observed in the present population (Table 3-4) deserve attention, as reduction of adiponectin has been reported to be associated with insulin resistance (Yadav et al., 2013). Inverse association of blood Hg with serum adiponectin, which was observed together with the positive association between blood Hg and HOMA-IR, should be interpreted that insulin resistance by Hg exposure could be to in part explained by the changes in adiponectin. Adipokines have a regulatory role in the mechanism of insulin resistance. Reduction of adiponectin has been reported to be associated with IR, and the net action of leptin has been reported to inhibit appetite and decrease glucose (Yadav et al., 2013). However, only limited epidemiological information is available on the relationship between metal exposure and changes in adiponectin. The association between metal exposure and adipokines appears to vary by types of metal and adipokines along with demographic factors such as age and sex. In a Mexican birth cohort, higher total metals were associated with higher adiponectin in Bayesian Kernel Machine Regression model (Kupsko et al., 2018). In 64-year old women in Sweden (n = 590), serum adiponectin did not show significant correlations with blood and urinary cadmium (Barregard et al., 2013). In a Canadian cohort of mother-infant pairs (n = 1188), maternal exposure to Hg, Cd, Pb, and As was not significantly associated with adiponectin measured in fetal umbilical cord blood. In contrast, increased OR of high leptin (≥90%) was found in the highest quartile of maternal blood Cd only among males (n = 639) (Ashley-Martin et al., 2015).

Significant positive association between blood Hg and serum GGT, which was independent of current alcohol consumption (Table 3-5), supports in part the link between Hg exposure and IR. Serum GGT has been suggested as an independent risk factor of T2DM or cardiovascular disease, regardless of alcohol consumption, as well as a marker of oxidative stress (Lee et al., 2004). The positive association between Hg exposure and serum GGT

observed in the present study is in line with observations of previous studies (Seo et al., 2014; Dierickx, 1980). In Korean adults from KNHANES 2010 data (n = 1959), a significant positive association between blood Hg and serum GGT was reported in both sexes (Seo et al., 2014). Experimental study also supports the relationship. Increased urinary GGT activity was observed in male Sprague-Dawley rats, in Hg treated group, but not in Pb and Cd treated groups (Dierickx, 1980).

3.4.3. Mediation effects of adiponectin and GGT

It was found that the association between Hg and IR was mediated by serum adiponectin and GGT among the women of reproductive age. In the subjects of the current study, several markers such as adiponectin, obesity, GGT, and IR are closely linked with each other (Fig. 3-1), and because one outcome can be on a causal pathway to another outcome, mediation analysis was conducted. A couple of studies reported mediation effects of oxidative stress markers in the association between phthalate exposure and IR or DM (Dong et al., 2018; Li et al., 2019), but no studies have been done for heavy metals. To our knowledge, this is the first study, which reports significant indirect effects of adiponectin and GGT in the association between blood Hg and IR. Our finding implies one of possible mechanisms of IR induced by Hg exposure.

I considered the relationship between variables to build a directed acyclic graph (DAG). We checked if one metabolic marker could precede others. There are several reports in literature that support mediation effects of adiponectin and GGT on the association between chemical exposure and IR. Serum adiponectin is inversely associated with IR (Yadav et al., 2013). A decline in adiponectin precedes IR (Stefan, 2002; Yamamoto et al., 2004). GGT, a non-specific marker of oxidative stress (Lee et al., 2004), predicts reduced insulin sensitivity, which might be related to hepatic IR (Thamer et al., 2005). In addition, GGT has also been

reported to be a significant mediator of the association between phthalate exposure and IR (Dong et al., 2019). Although central obesity is an important risk factor for metabolic syndrome and IR (Hardy et al., 2012), whether obesity precedes IR or vice versa is a matter of debate (Erion and Corkey, 2017), and therefore we did not include obesity markers as mediators in the association. Obesity, IR, and related health parameters are closely associated with other. Therefore, further cohort or experimental studies are needed to identify etiology of these health outcomes.

3.4.4. Strengths and limitations

Among women of reproductive age, blood Hg at its current level of exposure was associated with higher BMI and higher IR, and decreased adiponectin was identified as a potential mediator of IR induced by Hg exposure. As a cross-sectional study, causal inference cannot be made based on this study. Moreover, because our study population included only adult women aged between 20s and 40s recruited from medical institutes, it may not represent the adult Korean population. However, `our observation provides reliable information for adult women before menopause, on possible effects of major metals on metabolic markers, considering that age and sex are important factors related to obesity and IR.

Chapter 4 Complex chemical exposure profile among adult women before menopause and its association with obesity and metabolic markers

4.1 Introduction

Humans are exposed to a myriad of chemicals in daily life. These multiple chemicals, as a whole or in parts, may influence development of diseases. Traditional approach of environmental epidemiology studies on chemicals had focused on exposure to single or a limited number of chemicals. Recently, there is a growing consensus that such approach may result in false conclusions, and models that incorporate multiple chemicals relevant to a given exposure scenario is warranted (Kalloo et al., 2018; Lee et al., 2017; Robinson et al., 2015). For example, the concept of exposome which comprises a totality of exposure that an individual experiences throughout life courses (Wild, 2005; Wild, 2012) echoes realization of such limitation.

Several analytical approaches have been applied to incorporate multiple chemicals in the association studies. One such example is the principal component analysis (PCA) (Kalloo et al., 2018; Lee et al., 2017; Robinson et al., 2015). PCA is dimension reduction method that converts data into linearly uncorrelated principal components, while retaining most of the variation of the data (Hatcher, 1994). Recently, several other approaches such as elastic net regression, weighted quantile sum regression (WQSR) and Bayesian kernel machine regression (BKMR) have been proposed to include multiple pollutants in the models (Bobb et al., 2015; Carrico et al., 2015; Czarnota et al., 2015; Lenters et al., 2015). Elastic net regression is a penalized regression model to select variables after controlling for

multicollinearity among variables (Zou and Hastie, 2005). WQSR is a method to derive a weighted index that estimates the mixed effect of multiple predictor variables on a given outcome, and was reported to have improved accuracy in models compared to traditional regression or shrinkage methods (Carrico et al., 2015). BKMR is proposed as a useful method to estimate joint effects of multiple pollutants and allows nonlinear relationship (Bobb et al., 2015). While each of these methods has advantages, these methods have shown rooms to improve. Elastic net regression was less efficient to select chemicals related to outcomes compared to WQSR (Czamota et al., 2015). WQSR provides weights with nonzero, and therefore, a choice of threshold is needed to select variables, but this choice can be challenging (Carrico et al., 2015). In case of lack of co-exposure patterns, the BKMR estimates have to rely on extrapolation (Cocker et al., 2018).

In previous chapters, it was demonstrated that several environmental chemicals are associated with metabolic markers in adult women before menopause. In the present chapter, the aim is to show that chemical exposure profile among the women is highly complex. In addition, I intend to demonstrate that the association model that incorporate multiple chemicals that are measured in urine (Chapter 2), blood (Chapter 3), and serum can be developed to identify potential chemical determinants of obesity or metabolic markers.

4.2 Materials and methods

4.2.1 Study population

Initial population comprises of the women of reproductive age (20-48 years old) (n = 516) recruited from five university hospitals located in Seoul, Incheon, Ansan, and Jeju, South Korea between 2015 and 2016. Details about the participating women were descripted in Chapters 2 and 3.

Among the initially recruited women, two participants who had fasting glucose measurements ≥126 mg/dL, and 33 participants who were pregnant at the time of recruitment were excluded. Participants with missing information on age, BMI, urinary nicotine metabolite level, and/or current alcohol consumption habits were also excluded. Lastly, participants who were measured for urinary phthalate metabolites and environmental phenols, blood heavy metals, and serum persistent organic pollutants (POPs) were chosen, and the final number of subjects of overall multi-pollutants was 104. Informed consent was obtained from all participants. This study was approved by the Institutional Review Board of the School of Public Health, Seoul National University (IRB No. 1509/001-011).

4.2.2 Measurement in urine and blood samples

Health effect markers

Urinary creatinine, serum glucose, and serum insulin were measured by a commercial laboratory (Green Cross LabCell, Yongin, Korea). Serum adiponectin and leptin were measured using an enzyme-linked immunosorbent assay kit (Duoset, R&D Systems, Minneapolis, MN, USA). Insulin resistance status was estimated using the homeostatic model assessment for insulin resistance (HOMA-IR) and was calculated as fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5 (Wallace et al., 2004).

4.2.3 Statistical analysis

Linear regression analyses were conducted to analyze associations of serum POPs concentrations with adiponectin, leptin, and γ-glutamiltransferase (GGT). Due to right skewness, GGT, insulin, HOMA-IR, and chemical concentrations were ln-transformed before conducting linear regression. In the crude model, no covariates were adjusted. In the adjusted model, covariates were chosen based on a previous study which reported possible risk factor of T2DM (Zheng et al., 2018), and were included in the linear regression model. These covariates included age (categorical: 25-29, 30-39, 40-48 years), urinary nicotine metabolite (categorical: <10, 11-499, ≥500 ng/mL), and current alcohol drinking (categorical: drinker and non-drinker).

Linear regression analyses were conducted to evaluate the associations of multi-pollutants measured in urine, blood, and serum, with obesity and metabolic markers. To evaluate overall multiple chemical exposures simultaneously, principal component analysis (PCA) with varimax rotation was conducted. For urinary chemicals, creatinine-corrected values were used to adjust urine dilution. Creatinine-corrected molar concentrations of urinary chemicals and molar concentrations of blood and serum chemicals were ln-transformed. Firstly, PCA was conducted with no constraints on the total number of principal components. By examining Scree plots and selecting a number of principal components that explained ≥50% of the variance in the data, total six principal components were restricted. Models that included all six factors separately and simultaneously were conducted.

A value of $p<0.05$ was considered to be significant. All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA) except the Spearman correlation analyses. The correlation matrix was calculated using R version 3.5.1 and visualized using the R package ‘corrplot’.

4.3 Results

4.3.1 Characteristics of study population

Participating women were aged between 25 and 48 years, with a majority in thirties (61.5%) (Table 4-1). Most participants (43.3%) showed normal range of BMI (18.5-22.9 kg/m^2), with 2.9% and 5.8% of the participants in underweight (<18.5 kg/m^2) and moderate obese (≥30 kg/m^2), respectively.

Table 4-1. Characteristics of study population (n = 104).

	N (%)
Age	
25-29	11 (10.6)
30-39	64 (61.5)
40-48	29 (27.9)
BMI (kg/m^2)	
<18.5	3 (2.9)
18.5-22.9	45 (43.3)
23-24.9	23 (22.1)
25-29.9	27 (26.0)
≥30	6 (5.8)
Urinary nicotine metabolite (ng/mL)	
<11	92 (88.5)
11-500	6 (5.8)
≥500	6 (5.8)
Current alcohol consumption	
Drinker	70 (67.3)
Non-drinker	34 (32.7)
	Median (25^{th}-75^{th})
Health effect markers	
Adiponectin (μg/mL)	6.3 (5.1-7.4)
Leptin (ng/mL)	10.9 (6.3-17.7)
GGT (U/L)	12 (10-18)
Fasting glucose (mg/dL)	89 (86-95)
Fasting insulin (μU/mL)	7.1 (5.0-10.2)
HOMA-IR	1.5 (1.1-2.2)

4.3.2 Profiles of chemical mixture exposure among women of reproductive age

The analytical results on urine, whole blood, and serum samples showed that the chemical exposure profile among the participating women is complex (Fig. 4-1). Urinary concentrations of parabens occupied the greatest proportion among the measured chemicals: In pie graphs drawn with either 50^{th} or 95^{th} percentile exposures (total molar sum of a given chemical group), parabens showed > 60% of the total. Without parabens, the molar sums of phthalate metabolites and heavy metals from the population from whom such data are available (Chapters 2 and 3, n = 455) were 63% and 28%, respectively, when the 50^{th} percentiles of chemicals were considered. When only those with all chemical measurements are available (n=104), similarly, percentages of molar sum of phthalates and heavy were 60% and 32%, respectively.

The sum of quartiles of molar sum of each chemical group shows complexity of chemical exposure profile of the participating women (Fig. 4-2). This is a simplified presentation of the exposure profile because chemical measurements were converted to values between one and four according to quartiles of molar sum of each chemical group. No PBDEs showed detection frequency greater than 50%, and therefore, zero to 2 was assigned. Total sum of quartiles varied between 15 and 37, out of the theoretical range of variation between 9 and 39.

The percentage relative standard deviation (%) for each chemical ranged from 35.6% for Pb to 812.8% for TCS (Fig. 4-3).

Correlations between molar concentrations of chemicals were explored (Fig. 4-4). Creatinine-corrected molar concentrations of urinary chemicals, i.e., phthalates, parabens, benzophenones, and bisphenols, showed low to moderate levels of correlation. For POPs, PCB153, PCB180, and HCB showed high positive correlation, and PFCs also showed high

positive correlation. Meanwhile, PCB52 showed moderate negative correlations with PCB153, PCB180, and HCB.

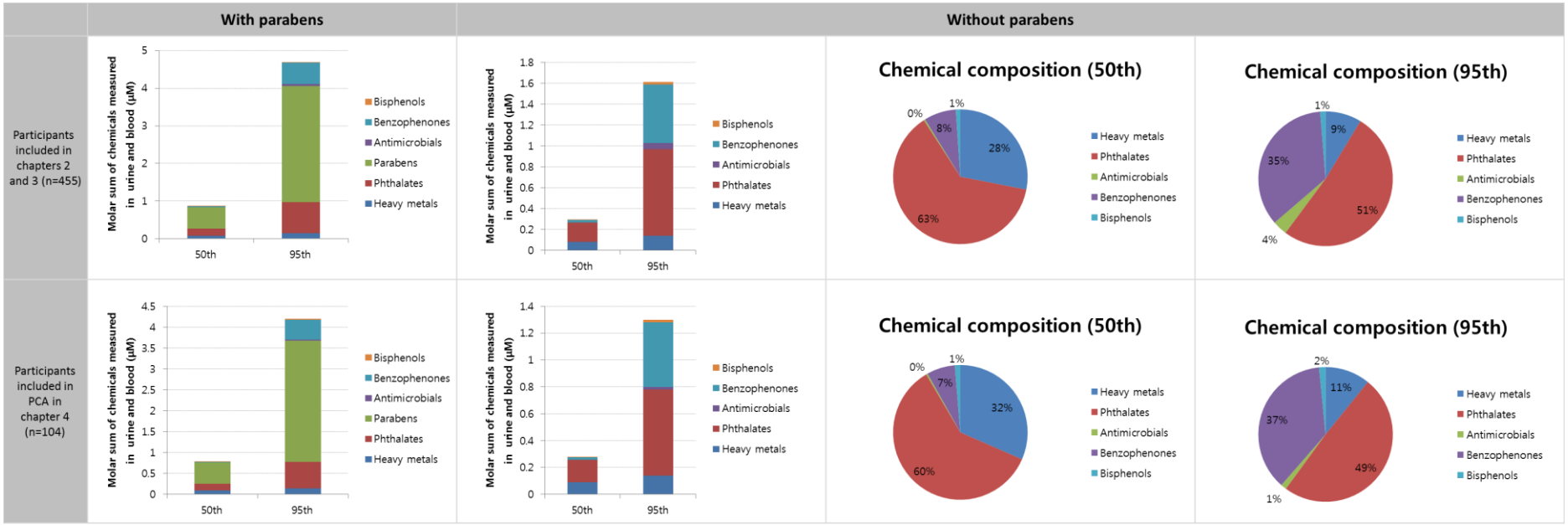

Fig. 4-1. Comparison of chemical composition used in chapters 2 and 3 (n = 455) and chapter 4 (n = 104). 50th and 95th percentiles of molar sum of chemical groups including and excluding parabens were suggested.

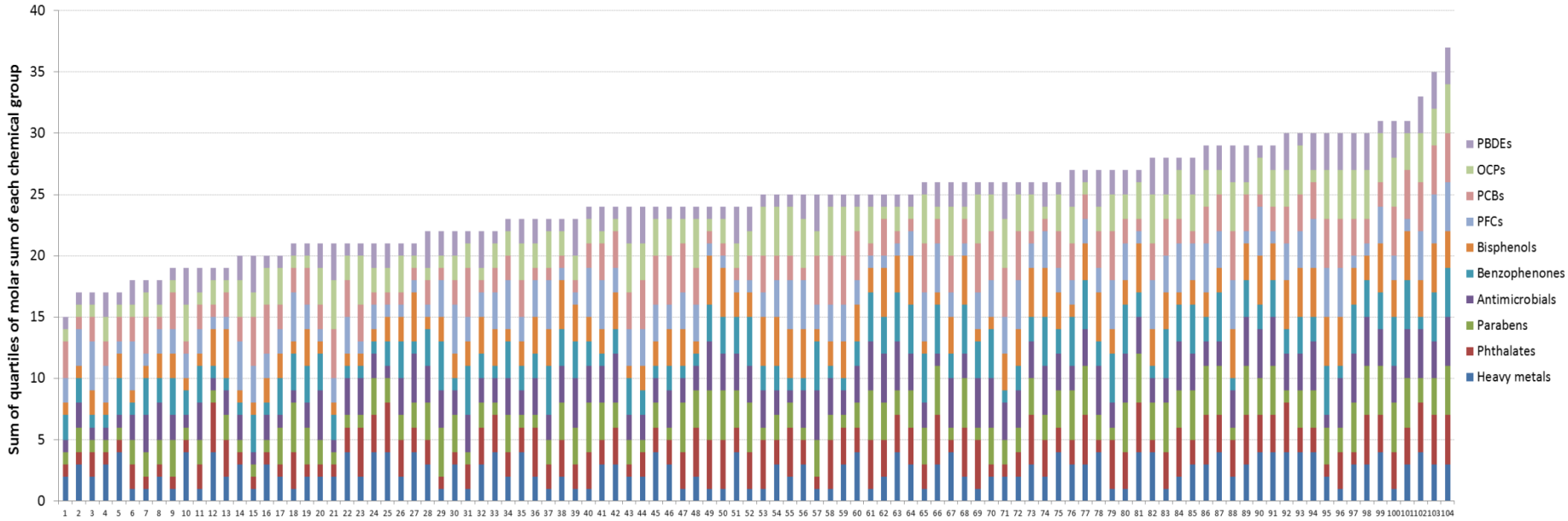

Fig. 4-2. Sum of quartiles of molar sum of each chemical group (n = 104). X axis indicates individual participants.

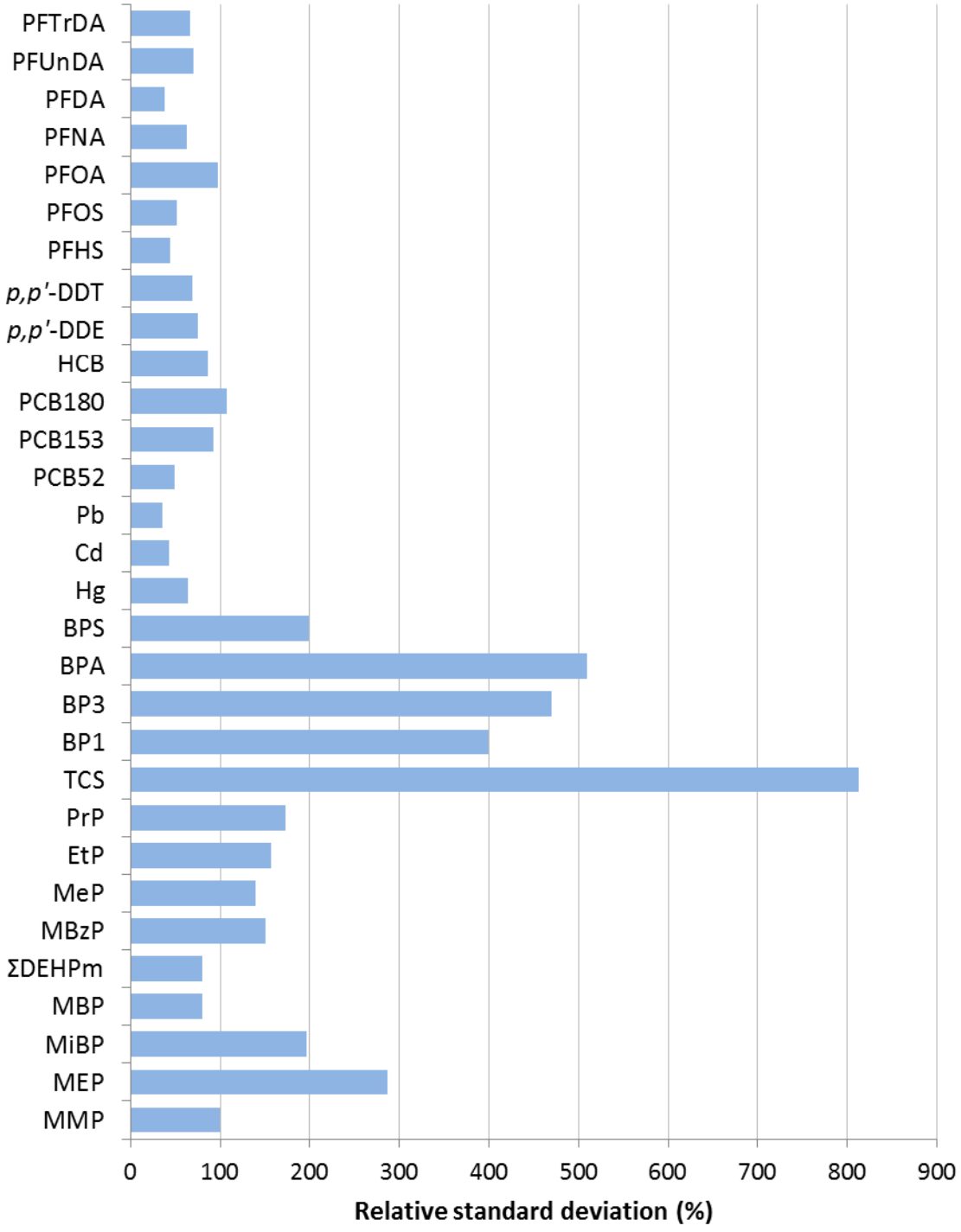

Fig. 4-3. Relative standard deviation for molar concentration of chemicals (n = 104).

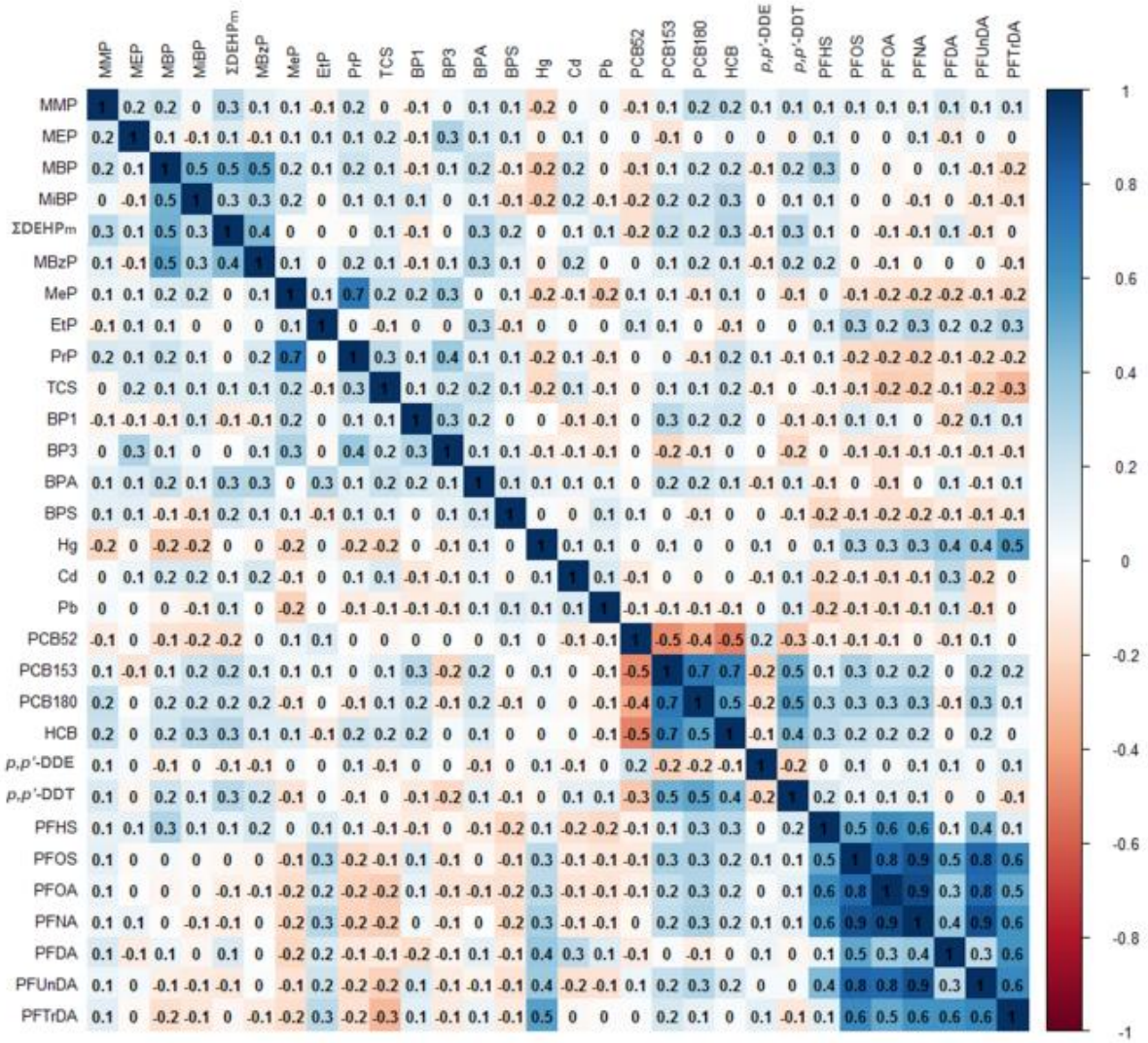

Fig. 4-4. Spearman's correlation of molar concentrations of chemicals (n = 104). Molar concentrations of urinary chemicals were creatinine-corrected.

4.3.3 PCA components results of multiple chemicals

Following a PCA on all measured chemical concentrations, six principal components were identified which accounted for 17.3%, 11.5%, 7.8%, 7.0%, 6.1% and 5.3% of the total variance (Table 4-2). Factor 1 was characterized by high loading of all PFCs, i.e., serum PFHS, PFOS, PFOA, PFNA, PFDA, PFUnDA, and PFTrDA. This factor was referred to as the PFC factor. Factor 2 exhibited high loading of PCB153, PCB180, HCB, and *p,p'*-DDT, and was referred to as PCB and OCP factor. Factor 3 had high loading of MeP, PrP, BP1, and BP3. This factor was referred to as environmental phenols factor excluding bisphenols. Factor 4, phthalate metabolite factor, had high loading of MBP, ΣDEHPm, and MBzP. Factor 5 had positive loading of BPA and BPS and negative loading of PFHS. Factor 6 was characterized with high loading of PCB52 and *p,p'*-DDE, which did not show high loading in Factor 2.

Table 4-2. Summary of the rotated factor pattern (n = 104).

	Factor1	**Factor2**	**Factor3**	**Factor4**	**Factor5**	**Factor6**
MMP	0.094	0.161	-0.009	0.420	0.164	0.329
MEP	0.031	-0.086	0.041	0.177	-0.078	0.313
MBP	-0.004	-0.045	0.139	**0.780**	-0.265	0.020
MiBP	-0.120	0.301	0.186	0.319	-0.126	-0.119
ΣDEHPm	-0.042	0.308	-0.056	**0.646**	0.180	0.224
MBzP	-0.063	0.162	0.202	**0.607**	0.148	0.106
BPA	0.101	0.115	0.283	0.277	**0.592**	0.035
BPS	-0.142	0.030	0.098	0.025	**0.590**	0.206
MeP	-0.105	-0.023	**0.784**	0.083	-0.113	0.047
EtP	0.361	-0.166	0.222	0.106	0.085	0.136
PrP	-0.142	-0.008	**0.719**	0.224	-0.033	-0.045
TCS	-0.162	0.223	0.287	0.120	0.087	-0.094
BP1	0.099	0.323	**0.519**	-0.358	0.191	0.063
BP3	-0.039	-0.070	**0.668**	-0.038	0.095	0.030
Hg	0.441	-0.106	-0.160	-0.091	0.333	-0.228
Cd	-0.018	-0.025	-0.145	0.431	0.240	-0.271
Pb	-0.003	-0.166	-0.332	0.075	0.420	-0.106
PCB52	-0.003	-0.103	-0.049	-0.031	0.074	**0.814**
PCB153	0.232	**0.860**	0.117	0.023	0.017	-0.144
PCB180	0.167	**0.785**	-0.120	0.086	-0.143	0.184
HCB	0.127	**0.751**	0.215	0.086	0.035	-0.185
p,p'-DDE	0.053	0.104	0.016	-0.005	0.046	**0.635**
p,p'-DDT	-0.063	**0.593**	-0.288	0.186	0.002	0.224
PFHS	**0.509**	0.159	0.074	0.188	**-0.581**	0.151
PFOS	**0.870**	0.206	-0.042	0.004	-0.153	0.047
PFOA	**0.836**	0.210	-0.054	-0.132	-0.193	0.098
PFNA	**0.898**	0.182	-0.063	-0.054	-0.169	0.124
PFDA	**0.598**	-0.230	-0.193	0.394	0.204	-0.195
PFUnDA	**0.834**	0.139	-0.115	-0.164	-0.139	0.102
PFTrDA	**0.777**	-0.049	-0.122	0.029	0.238	-0.146
Eigenvalues	5.18	3.45	2.34	2.08	1.81	1.60
Variance explained (%)	17.3	11.5	7.8	7.0	6.1	5.3
Cumulative variance (%)	17.3	28.8	36.6	43.5	49.6	54.9

Bold numbers indicate factor loading >|0.5| (Hair et al., 1998).

All molar concentrations were ln-transformed.

Molar concentrations of chemicals measured in urine were creatinine-corrected.

4.3.4 Associations with obesity and metabolic markers

In the single factor model, Factor 4 ($R^2_{partial}$ = 0.077), characterized by phthalate metabolites, was significantly positively associated with adiponectin (Table 4-3). Factor 4 ($R^2_{partial}$ = 0.051) also showed significant positive association with fasting glucose. These observations are similar to the significant positive associations of several phthalate metabolites with serum adiponectin or fasting glucose which were observed in Chapter 2. Factor 1 ($R^2_{partial}$ = 0.049) characterized by PFCs showed marginally significant (p<0.1) association with percent body fat.

Multi-factor model which was developed with six factors together showed similar results (Table 4-3). Factor 4 ($R^2_{partial}$ = 0.077) showed significant positive associations with adiponectin. Factor 4 ($R^2_{partial}$ = 0.053) showed significant positive association with fasting glucose.

Table 4-3. Associations of chemical exposure factors with obesity and metabolic markers (n = 104).

	Single factor			**Multi-factor**		
	β (95%CI)	***p*-Value**	**$R^2_{partial}$**	**β (95%CI)**	***p*-Value**	**$R^2_{partial}$**
BMI						
Factor1	0.092 (-0.666, 0.850)	0.810	0.001	0.086 (-0.688, 0.860)	0.826	0.001
Factor2	-0.060 (-0.789, 0.669)	0.871	0.000	-0.047 (-0.792, 0.698)	0.901	0.000
Factor3	0.007 (-0.710, 0.725)	0.984	0.000	0.007 (-0.725, 0.738)	0.986	0.000
Factor4	0.107 (-0.624, 0.838)	0.772	0.001	0.110 (-0.637, 0.857)	0.771	0.001
Factor5	-0.092 (-0.803, 0.620)	0.799	0.001	-0.094 (-0.818, 0.631)	0.798	0.001
Factor6	0.433 (-0.270, 1.136)	0.224	0.015	0.433 (-0.288, 1.154)	0.236	0.015
Percent body fat (n = 69)						
Factor1	1.756 (-0.169, 3.682)	0.073	0.049	1.557 (-0.525, 3.638)	0.140	0.037
Factor2	-1.153 (-2.805, 0.500)	0.168	0.029	-0.686 (-2.434, 1.061)	0.435	0.010
Factor3	-0.758 (-2.375, 0.859)	0.353	0.014	-0.870 (-2.474, 0.734)	0.282	0.020
Factor4	0.871 (-0.613, 2.355)	0.245	0.021	0.794 (-0.674, 2.263)	0.283	0.019
Factor5	-0.057 (-1.494, 1.381)	0.938	0.000	-0.102 (-1.517, 1.313)	0.886	0.000
Factor6	1.324 (-0.260, 2.909)	0.100	0.042	1.206 (-0.372, 2.785)	0.132	0.038
Ln-GGT						
Factor1	-0.002 (-0.121, 0.117)	0.970	0.000	0.003 (-0.117, 0.123)	0.963	0.000
Factor2	0.065 (-0.049, 0.178)	0.260	0.013	0.067 (-0.049, 0.182)	0.255	0.014
Factor3	-0.025 (-0.138, 0.087)	0.657	0.002	-0.025 (-0.138, 0.089)	0.666	0.002
Factor4	0.030 (-0.084, 0.145)	0.598	0.003	0.035 (-0.082, 0.151)	0.557	0.004
Factor5	0.026 (-0.085, 0.138)	0.641	0.002	0.025 (-0.088, 0.137)	0.666	0.002
Factor6	0.062 (-0.048, 0.172)	0.267	0.012	0.062 (-0.050, 0.174)	0.274	0.013
Adiponectin						
Factor1	0.029 (-0.352, 0.410)	0.882	0.000	0.056 (-0.317, 0.430)	0.765	0.001
Factor2	-0.030 (-0.396, 0.336)	0.871	0.000	0.004 (-0.356, 0.364)	0.983	0.000
Factor3	-0.044 (-0.405, 0.316)	0.808	0.001	-0.042 (-0.395, 0.311)	0.813	0.001
Factor4	0.510 (0.157, 0.863)	**0.005**	0.077	0.510 (0.149, 0.871)	**0.006**	0.077
Factor5	0.081 (-0.276, 0.438)	0.653	0.002	0.075 (-0.275, 0.425)	0.670	0.002
Factor6	-0.204 (-0.557, 0.149)	0.255	0.013	-0.204 (-0.552, 0.144)	0.247	0.014
Leptin						
Factor1	-1.020 (-2.794, 0.753)	0.256	0.013	-1.075 (-2.882, 0.732)	0.241	0.015
Factor2	0.197 (-1.519, 1.913)	0.820	0.001	0.084 (-1.654, 1.822)	0.924	0.000
Factor3	0.297 (-1.392, 1.986)	0.728	0.001	0.325 (-1.382, 2.031)	0.707	0.002
Factor4	-0.949 (-2.661, 0.763)	0.274	0.012	-0.986 (-2.729, 0.758)	0.265	0.013
Factor5	0.258 (-1.417, 1.932)	0.761	0.001	0.251 (-1.441, 1.942)	0.769	0.001
Factor6	0.611 (-1.051, 2.274)	0.467	0.005	0.623 (-1.060, 2.305)	0.464	0.006
Fasting glucose						
Factor1	0.005 (-1.607, 1.616)	0.996	0.000	0.108 (-1.477, 1.693)	0.893	0.000

Factor2	0.012 (-1.537, 1.561)	0.988	0.000	0.127 (-1.397, 1.651)	0.869	0.000
Factor3	-0.920 (-2.434, 0.594)	0.231	0.014	-0.905 (-2.402, 0.592)	0.233	0.015
Factor4	1.766 (0.252, 3.280)	**0.023**	0.051	1.768 (0.239, 3.298)	**0.024**	0.053
Factor5	0.314 (-1.197, 1.825)	0.681	0.002	0.284 (-1.200, 1.767)	0.705	0.002
Factor6	1.089 (-0.400, 2.578)	0.150	0.021	1.089 (-0.387, 2.564)	0.146	0.022
Ln-insulin						
Factor1	0.008 (-0.118, 0.135)	0.895	0.000	0.007 (-0.120, 0.135)	0.908	0.000
Factor2	-0.053 (-0.174, 0.068)	0.386	0.008	-0.055 (-0.178, 0.068)	0.375	0.008
Factor3	-0.043 (-0.162, 0.077)	0.479	0.005	-0.043 (-0.164, 0.077)	0.476	0.005
Factor4	-0.011 (-0.133, 0.111)	0.858	0.000	-0.016 (-0.139, 0.107)	0.798	0.001
Factor5	0.079 (-0.039, 0.196)	0.188	0.017	0.080 (-0.040, 0.199)	0.190	0.018
Factor6	0.048 (-0.070, 0.165)	0.424	0.006	0.047 (-0.072, 0.166)	0.435	0.006
Ln-HOMA-IR						
Factor1	0.007 (-0.128, 0.143)	0.914	0.000	0.008 (-0.129, 0.144)	0.913	0.000
Factor2	-0.053 (-0.183, 0.077)	0.418	0.007	-0.054 (-0.185, 0.077)	0.417	0.007
Factor3	-0.053 (-0.181, 0.074)	0.410	0.007	-0.054 (-0.183, 0.075)	0.410	0.007
Factor4	0.008 (-0.123, 0.139)	0.904	0.000	0.003 (-0.129, 0.135)	0.965	0.000
Factor5	0.082 (-0.044, 0.208)	0.202	0.016	0.082 (-0.046, 0.210)	0.204	0.017
Factor6	0.059 (-0.067, 0.185)	0.357	0.009	0.058 (-0.069, 0.185)	0.368	0.009

In all models, age, urinary nicotine metabolite, and current alcohol consumption were adjusted.

Bold numbers indicate statistically significance (p<0.05).

4.4 Discussion

Complex chemical profile observed in the present women population outlines that multiple chemicals should be considered when developing association models with health outcomes related to chemical exposure. The results of PCA (Table 4-2) show that complex chemical exposure profile developed from measurement of diverse biological media could be grouped into several groups of chemicals or factors. Interestingly, each factor is highly loaded with similar group of chemicals. Two major factors dominated variance were highly loaded with POPs, Factor 1 with PFCs, and Factor 2 with PCB153, PCB180, HCB, and *p,p'*-DDE. Factors 3 and 4 were highly loaded with urinary environmental phenols and phthalates, respectively, and Factor 5 were positively loaded with bisphenols and negatively loaded with PFHS. These observations suggest that these groups of chemicals are likely to be independent at least in terms of exposure sources or pathways.

One observation that is noteworthy is that PCB52 is loaded in Factor 6, but other higher chlorinated PCBs, e.g., PCB153 and PCB180, loaded in Factor 2. This observation might be due to difference in major exposure sources of this group of chemicals. In general, higher levels of PCB153 and PCB180 are detected in food (Bernard et al., 1999; Schecter et al., 2010). Because lower chlorinated PCBs such as PCB52 were relatively mobile and used in sealant material, higher levels are detected in the air compared to higher chlorinated PCBs (Bogdal et al., 2013; Schettgen et al., 2012).

Urinary chemicals such as phthalates and benzophenones showed low to moderate correlations (Fig. 4-4). Several serum organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and perfluorinated compounds (PFCs) showed moderate to high correlations within the same chemical classes. In particular, PFOS, PFOA, PFNA, and PFUnDA are highly correlated with rho greater than 0.8, which was similar to a previous

study (Haug et al., 2009). High correlations observed among certain chemicals in the present population may reflect that these chemicals share common exposure sources such as food, drinking water, and air (Jian et al., 2017).

It is noteworthy that the significant associations detected between factors and target health markers using PCA were comparable to those identified in Chapters 2 and 3. Similar to Chapter 2, Factor 4 (phthalate factor) was significantly positively associated with adiponectin and fasting glucose. Metal factors were not found according to factor loading greater than 0.5 (Hair et al., 1998). However, when factor loading greater than 0.4 was employed, blood Hg, Cd, and Pb were relatively highly loaded in Factors 1, 4, and 5, respectively. Though some statistical significance of blood metals observed in Chapter 3 disappeared in its subpopulation observed in Chapter 4, blood Hg and Cd were still significantly positively associated with obesity markers and adiponectin, respectively. Considering positive association of Factor 1 with obesity marker, and positive association of Factor 4 with adiponectin, these two metals might influence the associations.

PCA is a dimension reduction approach that reduces the number of correlated variables into a smaller number of constructs (factors) which are uncorrelated with each other (Hatcher, 1994). Because chemicals which have common sources are likely to be correlated, PCA can reflect exposure patterns due to common sources. Principal components are constructed based on the correlation among the predictors regardless of the outcomes. Therefore, the significant associations observed with a couple of factors which are comparable to those observed in previous chapters provide additional line of evidence that supports the association of these chemicals with obesity or metabolic markers.

In the current study employing dozens of chemicals, it was found that factors identified from PCA based on molar concentrations are highly loaded with chemicals that belong to specific groups. More interestingly, it was found that some phthalates are positively

associated with adiponectin and fasting glucose, consistently by using both elastic net regression and PCA. Further validation of this observation in other populations is warranted.

Chapter 5 Summary and conclusions

The associations between multiple classes of chemicals and obesity and its related health effects among women of reproductive age were evaluated in a series of three studies. Humans are exposed to multiple chemicals simultaneously. Thus, chemicals with short to long half-lives in different biological matrix were measured and evaluated multi-pollutant models by adjusting chemical markers.

In Chapter 2, the associations between urinary non-persistent chemicals including phthalate metabolites and phenolics were assessed (n = 459). The same classes of chemicals showed moderate to high correlations. To select the most predictive variables to an outcome, elastic net penalized regression was conducted. MiBP, ΣDEHPm, BPA, and BPS may be the most predictive variables to obesity, fasting glucose, or insulin resistance in Chapter 2.

In Chapter 3, the associations between heavy metals, Hg, Cd, and Pb were assessed (n = 456). Higher blood Hg was associated with higher BMI, GGT, and HOMA-IR, and lower adiponectin. Significant indirect effects of GGT and adiponectin were found in the association between blood Hg and HOMA-IR. Increased odds ratio of HOMA-IR>75th percentile per quartile increase of blood Pb was found, but the association disappeared after adjusting blood Hg which showed significant correlation with blood Pb. Because Hg showed significant associations with several markers, mediation analysis was conducted. Significant mediation effects of adiponectin and GGT in the association between Hg exposure and HOMA-IR were found. The implication of this chapter is that possible mediators of IR induced by Hg exposure were suggested by conducting mediation analysis.

In the last chapter, all chemicals used in Chapter 2, 3, and 4 were included, and confirmed the robustness of analysis by performing principal component analysis (PCA).

Although PCA cannot identify factors related to an outcome, PCA can identify correlated exposures, i.e., common exposure sources or metabolic processes. Similar to Chapter 2, factor 4 characterized by phthalate metabolites showed positive significant associations with serum adiponectin and fasting glucose.

This study design is cross-sectional, and therefore, this study could not provide causal inference. Small sample size of POPs concentrations can be another limitation. However, information on exposure patterns including multiple chemicals is still limited, and therefore, this study implies that the associations of multiple chemicals with obesity and metabolic markers were assessed in women of reproductive age. Because similar chemical classes such as PFCs and phthalates share metabolic pathway or common sources, management is needed to reduce these correlated chemical groups as a whole.

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국문 초록(Abstract in Korean)

가임기 여성의 화학물질 복합노출과 대사 관련 지표와의 상관성

여러 내분비교란물질(endocrine disrupting chemicals, EDCs)은 비만, 당뇨 등 대사질환에 영향을 미치는 것으로 알려져 있다. 환경 역학에서는 내분비교란물질과 비만, 대사 지표와의 상관성에 관한 연구가 활발히 보고되고 있으나, 이는 비용 등의 문제로 인해 주로 한 매체에서 측정된 물질군으로 한정되어 있다. 또한 중요한 대사 관련 지표 중 하나인 아디포카인과의 상관성에 관한 연구는 부족한 실정이다.

본 연구에서는 여러 매체에서 측정된 내분비교란물질과 비만, 대사 지표와의 상관성을 보았다. Body mass index (BMI)와 체지방률을 비만의 지표로, 두 아디포카인, 즉 아디포넥틴과 렙틴, γ-glutamyl transferase (GGT), 공복 혈당, 인슐린, homeostatic model assessment for insulin resistance (HOMA-IR)을 대사질환 지표로 활용하였다. 대상인구 집단인 가임기 여성은 2015 년도와 2016 년도에 516 명 모집되었다. 소변, 전혈, 혈청 시료는 한 번씩 수거되었으며, 장(chapter) 별로 동일한 시료에서 측정된 물질과 건강영향 지표와의 상관성을 살펴보았다. 두 번째 장에서는 소변에서 측정된 프탈레이트 대사체와 환경성 페놀류의 비잔류성 화학물질을, 세 번째 장에서는 전혈에서 측정된 중금속을, 네 번째 장에서는 혈청에서 측정된 폴리염화 바이페닐(polychlorinated biphenyls,

PCBs), 유기염소계 살충제(organochlorine pesticides, OCPs), 폴리브롬화 다이페닐에테르(polybrominated diphenyl ether, PBDEs), 과불화화합물(perfluorinated compounds, PFCs)의 잔류성 유기오염물질을 살펴보았다. 또한 네 번째 장에서는 잔류성 유기오염물질뿐 아니라, 소변, 전혈, 혈청 중 분석된 물질군의 정보를 모두 가지고 있는 참여자에 한해, 다중 노출을 고려한 다중회귀선형분석을 수행하였다.

두 번째 장에서는 소변 중 비잔류성 화학물질인 프탈레이트 대사체와 환경성 페놀류와 비만, 대사 지표와의 상관성을 살펴보았다. 대부분의 물질들이 유의한 상관성을 보여, 건강 지표에 관한 가장 설명력 있는 변수를 선택하기 위해 elastic net penalized regression 을 수행하였다. 단일 노출과 다중 노출 모델에서 일관성 있게 유의한 상관성을 보인 물질들은 다음과 같다. Bisphenol S (BPS)와 비만, 렙틴은 유의한 양의 상관성을 나타냈으며, ethyl paraben (EtP)와 di(2-ethylhexyl) phthalate 대사체 합(ΣDEHPm)은 아디포넥틴과 유의한 양의 상관성을 나타냈다. 공복혈당의 경우, ΣDEHPm 와 유의한 양의 상관성을 나타냈으며, mono-isobutyl phthalate (MiBP)와 BPS 는 HOMA-IR 과 유의한 양의 상관성을 나타냈다. 이를 통해 MiBP, ΣDEHPm, BPA, BPS 는 비만, 공복 혈당, 혹은 인슐린 저항성에 대한 설명력이 높은 물질이라는 것을 확인할 수 있었다.

세 번째 장에서는 전혈에서 측정된 중금속, 즉 수은, 카드뮴, 납과 비만, 대사 지표와의 상관성을 살펴보았다. 수은은 BMI, GGT, HOMA-IR 과는 양의

상관성을 나타냈으며, 아디포넥틴과는 음의 상관성을 나타냈다. 수은이 여러 건강영향 지표와의 유의한 상관성을 나타내어, 이 관계를 이해하기 위해 매개효과 분석(mediation analysis)을 수행하였다. 수은과 HOMA-IR 사이의 관계에서 GGT 와 아디포넥틴의 유의한 간접 효과를 확인할 수 있었다. 납과 HOMA-IR 은 유의한 상관성을 나타냈다. 중금속과 비만, 대사 지표와의 상관성에 관한 연구는 여러 인구집단에서 보고된 바 있으나, 본 연구는 수은과 인슐린 저항성과의 관계에서 GGT, 아디포넥틴을 매개변인으로 제안하였다는 점에서 의의가 있다.

네 번째 장에서는 앞선 두 장에서 분석된 물질과 잔류성 유기오염물질을 모두 포함하여 주성분 분석(principal component analysis, PCA)을 수행하여 노출 형태를 살펴보고, 선택된 주성분과 대사 지표와의 상관성을 살펴보았다(n = 104). 주성분 분석법은 공통된 노출원별로 물질군을 살펴보는데 유용한 통계적 기법으로, 과불화합물과 프탈레이트 등 특징적인 주성분을 확인할 수 있었다. 두 번째 장 결과와 유사하게, 프탈레이트로 특징되는 factor 4 는 아디포넥틴, 공복혈당과 유의한 양의 상관성을 나타내었다. 비슷한 물질군들은 상관성이 높아, 개별적인 물질로 상관성을 분석하는 데는 제한점이 있으므로, 물질군을 함께 관리할 필요성이 있다.

본 연구에서는 여러 물질군과 비만, 대사 지표와의 상관성을 동일한 집단에서 살펴보았다는 이점이 있다. 본 연구를 통해 여러 물질군과 대사 지표와의 상관성을 확인할 수 있었으며, 특히 프탈레이트 대사체는 공복혈당, 아디포넥틴과

일관성 있는 유의한 양의 상관성을 가지는 것을 확인할 수 있었다. 본 연구에서 관찰된 물질들의 복합노출은 물질을 보정하는데 초점을 두었으며, 물질 간의 상호작용 등에 관한 영향은 여러 물질군 조합의 실험연구를 통해 확인할 필요성이 있다.

표제어: 복합 노출; 비만; 아디포넥틴; 렙틴; 공복 혈당; 인슐린 저항성; 프탈레이트; 페놀; 중금속; 잔류성 유기오염물질

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